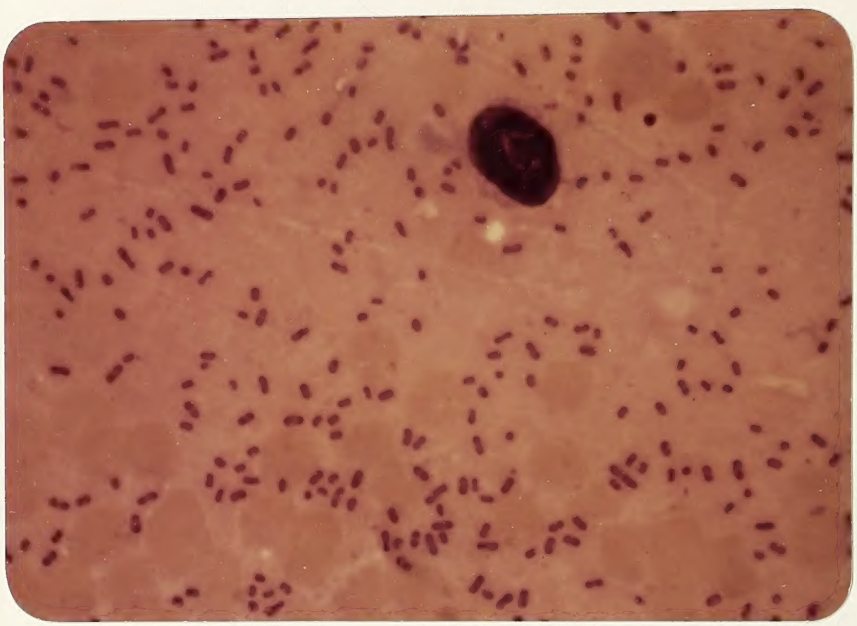
PLATE 1.



Giemsa-stained smear of heart blood of mouse with *Pasteurella multocida* infection

THE UNIVERSITY OF ALBERTA

PASTEURELLA MULTOCIDA IN HUMANS

Application of Serology
and the Methods of Numerical Taxonomy
to the Classification of Strains
and
A Consideration of Antibody as an Index of Infection

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

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DEPARTMENT OF BACTERIOLOGY

by

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ABSTRACT

A series of 59 human strains of Pasteurella multocida was investigated for the existence of sub-groups. Morphological, cultural and biochemical features of the strains were used to provide data for classification by the methods of numerical taxonomy. The degree of similarity between each possible pair of strains was determined by an electronic computer. The strains were sorted by the computer so that very similar strains were brought together to form groups. Human strains of P.multocida fell into four groups. Strains of group I were predominantly of respiratory origin and tended to produce a mucoid growth in culture. Group II strains, also recovered mainly from respiratory infection, were capsulated but did not produce a mucoid growth. Strains derived from dog and cat bite infections were non-capsulated and fell into groups III and IV. Distinct differences in fermentative properties were shown by strains of groups III and IV.

Serological procedures found applicable for classification of P.multocida were the indirect hemagglutination test and fluorescent antibody techniques. By means of the hemagglutination test, two serological types (Carter types A and D) were demonstrated, but almost 40% of the strains could not be classified due to absence of specific capsular antigens. Type A and D strains, most of which were recovered from respiratory infections, corresponded closely to group I and II respectively of the computer grouping.

Fluorescent antibody methods showed promise as a relatively simple means of demonstrating capsular types of P. multocida although further work is needed to determine the specificity of the procedure. Absorption of antisera for F.A. tests revealed the presence of at least

two sub-types within Carter types A and D.

The serological types of P. multocida isolated from human respiratory tract infections were found to be similar to those prevalent in cattle, pigs a nd poultry in Canada suggesting that these animals are the source of most human infection.

Serological investigations of patients with P.multocida infection indicated that high titers of antibody may be produced in respiratory infection. The presence of antibody to capsular substance was demonstrated by the hemagglutination test and antibody to somatic substance by agglutination tests with non-capsulated organisms.

A survey of 1000 sera from adult medical patients admitted to a local hospital showed an incidence of 1.6% positive for type A P.multocida. A high percentage of the patients with serum antibodies gave a history of recent contact with farm animals.

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Part I. Introduction and Literature Review

Introduction

Pasteurella multocida, under a variety of names, has been well known as an important pathogen of animals and birds for over three quarters of a century. However it is only within very recent years that this organism has become recognized as a not uncommon cause of human disease. Consequently, knowledge accumulated about this species has come almost exclusively from work with strains of animal origin. The morphological, cultural and biochemical features of animal strains are now well documented. Methods of typing have evolved which have yielded information concerning the inter-relationships of strains isolated from different animal hosts and have indicated an association of particular types with certain disease entities. Progress has been made in analysis of the important antigens of this species and at least two antigenic components have now been separated and characterized. Considerable research has been devoted to the immunological response of animals to various crude and partially purified preparations of Pasteurella multocida with a view to the development of suitable vaccines for the prevention of pasteurellosis in cattle, pigs and poultry.

In comparison very little is known about multocida strains of human origin. Numerous cases of human infection have now been documented but relatively little attention has been devoted to the responsible organism. It has not yet been determined unequivocally whether human strains are the same as those of animal origin or whether they possess distinguishing characters which set them apart. Only two attempts have been made to classify human strains and these studies yielded conflicting results. Further work is necessary to determine if the methods of typing found suitable for animal strains can be successfully applied to classification of strains of human origin. Little

is known about the antigenic make-up of these strains or about the immunological response of the human host to their presence.

The purpose of this study was to investigate some of these problems. It was proposed to study in detail a representative series of human strains of P. multocida for the existence of sub-groups and to determine the status of these strains in relation to those of animal origin. The morphological, cultural and biochemical features of the strains were examined and the information obtained used to provide data for classification by the methods of numerical taxonomy. Serological methods which have been applied to the classification of animal strains were investigated to select those suitable for further study. Certain drawbacks in the accepted methods led to the consideration of fluorescent antibody techniques as a possible alternative method of typing.

Serological studies of patients infected with P. multocida and of a series of "normal" hospital admissions were carried out to obtain information about the immunological response to infection and to gain some idea of the probable incidence of antibodies in the general population.

Review of Literature

Nomenclature

According to Topley and Wilson (1) the first member of the Pasteurella group was isolated by Kitt in 1878 from an epidemic disease affecting wild hogs and deer. Two years later, Pasteur obtained the causative agent from fowl cholera and in following years, organisms were isolated from swine plague, septic pleuropneumonia of calves and from diseases in other animals. A common feature of all these diseases was a haemorrhagic septicaemia. Likewise, the causative organisms resembled one another. All were short, bipolar staining Gram negative bacilli. In spite of these similarities the organisms were regarded as separate species and were named according to the animal from which they were derived; Pasteurella aviseptica from fowl, P. boviseptica from cows, P. suiseptica from pigs, P. lepiseptica from rabbits. The zoologic classification of Pasteurella was retained until about 20 years ago by which time the accumulation of evidence indicated that such a classification was meaningless. Investigation of the morphological, cultural, biochemical, serological and disease producing properties of strains from a variety of animal sources showed that these organisms were sufficiently similar to be considered a single species. Rosenbusch and Merchant, 1939 (2) recommended the name Pasteurella multocida first used by Kitt in 1885, while Topley and Wilson prefer Pasteurella septica.

Relationship with other members of the genus

The genus Pasteurella includes four well established species, P. pestis, P. tularensis, P. pseudotuberculosis and P. multocida. These organisms share morphological features and are all primarily pathogens of animals. However, P. multocida is not closely related to any of these other species. It does not show the fastidious nutritional requirements of P. tularensis but it is more exacting than the plague bacillus or P. pseudotuberculosis. Unlike the latter two organisms P. multocida fails to grow in the presence of bile salts. A very low degree of serological cross reactivity between P. multocida and P. pestis is reported by Larsen et al 1951 (3) but this is almost insignificant compared to the marked antigenic relationship between P. pestis and P. pseudotuberculosis. (4) P. multocida is not related antigenically to P. tularensis. Insect vectors play an important role in the transmission of plague and tularemia but there is no evidence that insects are ever involved in transmission of hemorrhagic septicemia or any of the other disease entities with which P.multocida is associated, Bain 1957 (5).

In view of the differences among present members of the Pasteurellae Meyer, 1958 (6) has predicted that eventually P.multocida will stand alone in the genus. If a new genus is created to include P.pestis and P.pseudotuberculosis the correct generic name for the new group would apparently be Yersinia (7).

Several less known and less well defined species are also listed under the Pasteurellae in Bergey's manual, 1957 (8). These include P.hemolytica, P.pneumotropica, P.pfaffi and P.novicida. P.hemolytica has been isolated from sheep and cattle and according to Carter, 1956 (9) is frequently associated with pneumonia or shipping fever in these animals.

Two cases of human infection have been reported, a cutaneous infection by Muraschi et al 1962, (10), endocarditis by Doty et al 1963, (11). The other species are more or less curiosities in which individual minor differences have been considered sufficient to warrant species rank. Such organisms continue to be reported.

Recently a Pasteurella-like organism isolated from human respiratory tract was described by Henriksen and Jyssum, 1960 (12). Although it resembled P.hemolytica in most characteristics a striking difference between the two organisms was the strong urease reaction given by the new isolate. For this reason the authors have proposed the name P.hemolytica var.ureae for the organism. Its possible role in human disease is not yet known.

Classification

Since the first of this century workers have attempted to classify the hemorrhagic septicemia bacilli by serological methods. Schutze, 1929, (13), reviewed much of the early work in which agglutination, agglutinin absorption and complement fixation procedures were employed. These studies and those of Yusef, 1935 (14) using precipitation tests showed that serological relationships did not correlate with host specificity. The existence of different serological varieties irrespective of animal origin was recognized but no satisfactory method of serological classification was evolved.

The first method of classification to receive general acceptance was that of Little and Lyon, 1943 (15). By means of a slide agglutination procedure these workers distinguished 3 types among the 30 cultures tested. Agglutination methods, however, are generally unsatisfactory because many capsulated strains are inagglutinable and many non-capsulated strains clump spontaneously in the presence of sodium chloride (16).

A more useful classification was introduced by Roberts in 1949 (17). He differentiated 4 types, I, II, III and IV among 37 strains by cross protection tests in mice.

Carter, 1952, (18) demonstrated a type specific, soluble, capsular antigen in P.multocida and on the basis of this antigen 3 different serological types were identified. The types designated A, B and C were distinguished by precipitation or capsular swelling tests. These procedures had shortcomings as routine typing techniques so Carter,1955 (19) investigated the application of indirect hemagglutination for classification of Pasteurella. He found that type specific substance released by

organisms at 56°C was adsorbed onto the surface of human red blood cells. The "coated" cells subsequently agglutinated in the presence of specific antisera. By means of the hemagglutination test Pasteurella multocida strains were divided into 4 types, A, B, C and D. These have been equated with Roberts type II, I, III and IV, respectively. An additional serotype E or V isolated in Central Africa is described by Hudson. (20)

Typing of strains from all over the world, either by the hemagglutination or cross protection techniques has revealed an association between serologic types and clinical manifestations in animals. (21) Classical hemorrhagic septicemia - a disease that results in great economic loss in cattle and buffalo in South-east Asia but is unknown on this continent - is caused by Type B (Roberts' Type I). Type A is the principle cause of fowl cholera and is frequently found complicating virus pneumonia in pigs. Type D occurs with greatest frequency in pigs but both A and D strains have been recovered from a variety of infections in different animals. Type C apparently occurs infrequently.

A number of workers have attempted to group P. multocida strains, on the basis of their fermentative ability. Rosenbusch and Merchant (2) divided 114 strains into 3 groups; Group I fermented arabinose and dulcite but not xylose, Group II attacked xylose but not arabinose nor dulcite, Group III were less uniform in their reactions. Roberts (17) noted that a correlation existed between his types as determined by cross protection tests and the fermentation of certain sugars. Type I strains failed to ferment arabinose but fermentation of this sugar was a characteristic of type II strains. Smith, 1958, (22) found an association between host species and some biochemical properties. Fermentation of mannitol was a feature of cat strains, fermentation of trehalose and maltose a characteristic of those from dogs. However, because of the variability in ferment-

ative properties among strains of P. multocida it is unlikely that a satisfactory classification on this basis can be achieved.

Antigenic Structure

In recent years progress has been made towards elucidating the antigenic structure of Pasteurella multocida. Carter & Annau in 1953 (23) showed that a type specific substance assumed to be of capsular origin was present in extracts of organisms heated to 56°C. From the extracts a serologically active polysaccharide (F substance) and a serologically inactive polysaccharide (M substance) were isolated. The M substance was depolymerized by hyaluronidase and was thus presumptively identified as hyaluronic acid.

Bain, 1959, (24) in his investigations of the antigenic structure of type I P. multocida found that even the gentlest extraction of cells yielded a multiplicity of antigens which could be demonstrated chemically in gel precipitation tests, or biologically by toxicity and hemagglutination. He believes that none of the antigens are exclusively somatic or exclusively capsular. Crude extracts were separable by orthodox chemical methods into polysaccharide, protein complex, and lipopolysaccharide, antigens. Attempts to purify these antigens have not as yet been entirely successful. "Purified" polysaccharide obtained by Knox and Bain, 1960, (25) after various fractionation procedures still produced 2 lines in gel diffusion tests. The polysaccharides were haptens that were capable of absorbing some of the mouse protecting capacity of immune serum.

Purification of the protein fraction has not been achieved (21). Crude protein preparations were highly immunogenic in animals indicating an important role for this fraction in inducing formation of protective antibodies.

Lipopolysaccharides obtained by phenol extraction of P.multocida cells have been described by MacLennan and Rondle, 1957, (26) and Bain and Knox, 1961, (27). Heptose was found to be a characteristic component of

the preparations. The similarity of this antigen to the endotoxin of other Gram negative bacteria was indicated by its pyrogenicity and toxicity to rabbits. The lipopolysaccharide was antigenic but was only partially protective to mice in active immunity tests. MacLennan and Rondle (26) found type specific lipopolysaccharides in P.multocida types I, IV, and V and Bain and Knox (27) have demonstrated that it is this antigen which absorbs onto the surface of red blood cells with resulting hemagglutination in the presence of specific antisera.

Gel diffusion tests indicate that the different immunological types of P.multocida have at least 6 antigens in common (28). These antigens are absent from other Pasteurella species.

Human Infection

Human infections with Pasteurella multocida are recorded before 1920 (13) but until recently infection in man was regarded as rare. A review of the literature from 1930-1947 by Schipper (29) listed only 39 cases that were supported by adequate bacteriological findings. Most of these occurred in Europe, the first reported case on this continent appearing in 1941. Within recent years, however, human infection is being recognized more frequently. Reports of 165 cases were made to the Public Health Laboratory Service in England during the three year period from 1957 - 1959 (30). In the Provincial Laboratory in Edmonton, Alberta, 71 instances of human infection have been discovered during the past five years. It is apparent, therefore, that P. multocida infection in man is not uncommon. The increase in observed cases is believed due to an increasing familiarity with the organism rather than to a marked rise in incidence of infection (21).

Human infections with P. multocida have been divided into 3 main groups. The best known of these is infection following animal bites or scratches. Dogs and cats are most frequently responsible but rabbits, panthers and even lions have been implicated. The second group consists of bronchial infections, mainly bronchiectasis or chronic bronchitis, with the organism being recovered from sputum or bronchial aspirate. The third group is composed of systemic infections appearing as meningitis, peritonitis, septicemia etc., Numerous examples of all these types of infections have been described in the literature (31,32,33,34,35).

It has not been established whether all human infections are of animal origin. Obviously the source in animal bite infection can be traced directly to the offending animal. Dogs and cats are frequently healthy carriers of P. multocida. Smith 1955 (36) reports that 61 of

111 dogs investigated harbored the organism in nose or tonsil. P.multocida was isolated from 9 of 14 healthy cats examined by Schenk 1938 (37). There is evidence that some systemic infections have also been derived from animal pets. For example, the child with meningitis described by Ewan, 1955 (38) had a pet kitten from which a strain of Pasteurella serologically identical to that from the child's CSF* was isolated. Similarly, in the case reported by Bearn et al, 1955 (39) of a woman with septicemia, strains of P. multocida culturally and biochemically identical were found in the patient's blood and in the throat of her pet cat.

But in the majority of systemic and bronchial infections the source of infection cannot be directly determined and is a matter of speculation. Olsen and Needham, 1952 (33) found that most of their patients with P. multocida associated with diseases of the respiratory tract, were farmers or of farming families. This suggests these infections may be derived from farm animals. But other cases of P. multocida infection have been reported in which there was no history of recent association with animals.

Establishment of the origin of human strains of P. multocida other than those found in animal bite infection must depend on some means of differentiating types within the species. By his hemagglutination test, Carter, 1962 (40) typed 71 human strains of P. multocida submitted to him by various workers across Canada. Strains from systemic and bronchial infections were mainly type A and D, the types common in pasteurellosis in cattle, pigs and fowl in this country. The majority of strains from dog and cat bites were non-typeable.

Using entirely different methods Talbot and Sneath, 1960 (7) in England investigated 59 strains of P. multocida, 52 of which were from human sources. On the principle that each feature of an organism is of equal taxonomic significance, a large number of characters of the strains

* cerebrospinal fluid

were compared by means of an electronic computer and the degree of similarity or divergence noted. They found that strains isolated from cat bite or scratch infections formed a characteristic group while dog strains were more variable. Most of the strains from internal lesions showed a similarity to the cat strains, which led these workers to conclude that the majority of human systemic and bronchial infections probably originate from cats. The discrepancy in the results of Carter in Canada and Talbot and Sneath in England has been explained by Stuart and Goodman(41) on the basis of the higher proportion of rural residents in this country but further investigation is needed to determine whether two such divergent methods of classifying the organisms are comparable at all.

Part II. Source, Isolation, Identification and Maintenance of Strains of Pasteurella Multocida Used in This Study.

SOURCE OF STRAINS

Of the total of 64 strains of Pasteurella multocida investigated 59 were of human origin, two came from dogs, and one each from cat, rabbit and buffalo. Six strains representing other species of Pasteurella were also studied for comparison. These included two strains of Pasteurella hemolytica, one of Pasteurella pseudotuberculosis and three Pasteurella-like organisms isolated from human sputum. The strains are listed in Table I.

The majority of the human strains of P. multocida were isolated from routine clinical material submitted to the Provincial Laboratory in Edmonton, over the seven year period from December 1955 to December 1962. The variety of source material represented by these strains is indicated in Table II.

ISOLATION

Primary isolation of most strains was obtained on nutrient agar with 5% sheep's blood. In 4 instances, P. multocida was isolated initially on blood agar plates incubated anaerobically as the corresponding aerobic plates were overgrown with other flora. On 3 occasions, primary growth was obtained in cooked meat only, the initial cultures on blood agar failing to grow. However, subsequent subculture of these strains on blood agar produced good growth.

Table 1.

Strains Investigated.

P. multocida - HUMAN ORIGIN

Number	Date of Isolation	Source	Clinical Data
1.	Dec. 1955	C.S.F.	meningitis
2.	Dec. 1955	C.S.F.	meningitis
3.	Oct. 1956	sputum	bronchiectasis
4.	Mar. 1958	thumb	cat bite
5.	July 1958	trachea	pneumonia
6.	July 1958	abdomen	peritonitis
7.	July 1958	wound	dog bite
8.	Aug. 1958	bronchial apsiration	bronchiectasis
9.	Oct. 1958	leg	cat bite
10.	Nov. 1958	ankle	cat bite
11.	Nov. 1958	sputum	asthma
# 12.	Nov. 1958	sputum	bronchiectasis
13.	Jan. 1959	sputum	chronic bronchitis
14.	Jan. 1959	leg stump	amputation site
15.	Feb. 1959	sputum	chronic bronchitis
16.	Apr. 1959	sputum	lobectomy - post op.
17.	Apr. 1959	leg	thigh abscess
18.	Apr. 1959	sputum	chronic bronchitis
19.	May 1959	sputum	bronchogenic cancer
20.	May 1959	sputum	bronchiectasis
21.	June 1959	sputum	chronic bronchitis
22.	Apr. 1959	sputum	bronchiectasis
23.	Apr. 1959	sputum	bronchiectasis
24.	Oct. 1959	sputum	bronchiectasis
25.	Jan. 1960	cheek	dog bite
26.	Mar. 1960	lower leg	cat bite
27.	Apr. 1960	sputum	bronchiectasis
28.	Aug. 1960	ear	ear discharge
29.	Sept. 1960	bronchial aspiration	bronchiectasis
30.	Sept. 1960	sputum	chronic bronchitis
31.	Oct. 1960	sputum	chronic bronchitis
32.	Feb. 1961	face	dog bite
* 33.	May 1961	wrist	cat bite
* 34.	Apr. 1961	sputum	pneumonia
35.	May 1961	ear	otitis media
36.	May 1961	hand	dog bite
37.	June 1961	sputum	bronchiectasis
38.	June 1961	cheek	dog bite
39.	July 1961	incision	no information
40.	Aug. 1961	sputum	chronic bronchitis

Table 1 (continued)

Number	Date of Isolation	Source	Clinical Data
41.	Aug. 1961	sputum	pneumonia
42.	Oct. 1961	sputum	chronic bronchitis
43.	Nov. 1961	sputum	chronic bronchitis
* 44.	Dec. 1961	?	no information
45.	Dec. 1961	sputum	chronic bronchitis
46.	Feb. 1962	sputum	bronchiectasis
47.	Feb. 1962	sputum	U.R.I. **
48.	Feb. 1962	leg	dog bite
49.	Feb. 1962	leg	dog bite
50.	Mar. 1962	sputum	chronic bronchitis
51.	Mar. 1962	sputum	asthma
52.	Mar. 1962	finger	wound
53.	Mar. 1962	throat	U.R.I.
54.	May 1962	right hand	dog bite
55.	June 1962	sputum	chronic bronchitis
56.	Sept.1962	peritoneal cavity	appendicitis
# 57.	Oct. 1962	sputum	bronchiectasis
58.	Dec. 1962	sputum	U.R.I.
59.	Dec. 1962	sputum	bronchiectasis

* Strains #33, 34 and 44 were received from the Calgary General Hospital. All other strains were isolated at the Provincial Laboratory, Edmonton.

** Undifferentiated respiratory infection

\# Strains 12 and 57 were isolated from the same patient in 1958 and 1962 respectively.

Table 1 (continued)

P. multocida - ANIMAL ORIGIN

Number	Date of Isolation	Source	Comment
60.	Jan. 1960	dog eye	dog that bit patient from which strain 25 isolated.
61.	June 1960	rabbit lung	respiratory infection
62.	March 1962	cat gums	gingivitis
63.		buffalo	type "B" strain from Ontario Veterinary College
64.		dog	type "C" strain from Ontario Veterinary College

Other Pasteurella strains

65.	June 1962	sheep	P.hemolytica - from Provincial Veterinary Laboratory, Edmonton
66.	June 1962	cow	P. hemolytica -from Provincial Veterinary Laboratory, Edmonton
67.			P.pseudotuberculosis Provincial Laboratory stock culture.
68.	June 1960	human sputum	Pasteurella-like organism
69.	Sept.1961	human sputum	Pasteurella-like organism
70.	June 1962	human sputum	Pasteurella-like organism

Table II.

Source of Human Pasteurella multocida Strains

Source	Number
Sputum or bronchial aspiration	34
Dog bite	8
Cat bite	5
Cerebrospinal fluid	2
Peritoneum	2
Ear	2
Amputation site	1
Abscess, wound or incision	3
Throat	1
Not known	1
	59

RECOGNITION AND IDENTIFICATION

The organisms were recognized initially by their characteristic colonial appearance, odor and microscopic morphology. (The features are described in detail later). Identification as Pasteurella multocida was confirmed by:

(a) failure of growth on MacConkey's agar
(b) sensitivity to penicillin
(c) fermentation of glucose and usually sucrose and mannitol.
(d) production of indol

MAINTENANCE

The only satisfactory method for long term storage proved to be lyophilization. Soon after isolation, a heavy suspension of each strain was prepared in 3% lactose and the material lyophilized. By this method the first strains isolated in this series have been successfully maintained up to the present time, a period of 8 years.

Cultures were maintained for shorter periods on tryptose agar slants with sufficient 2% serum broth added to prevent drying. The slants were prepared in 1/2 oz. round, screw-capped bottles. Most strains could be maintained at room temperature in this manner for two to three months but a few strains failed to survive for more than 1 - 2 weeks without subculture.

Lyophilization, or subculture on blood agar every three days, were the methods found satisfactory for maintaining the Pasteurella-like strains included in this study.

Part III. Classification of Pasteurella Strains by the Methods of Numerical Taxonomy.

INTRODUCTION:

Numerical or computer taxonomy is a relatively recent development in the field of microbial classification although the underlying concepts date back to Michel Adanson, an 18th century botanist. The principle, as outlined by Sneath, 1957 (42) is classification based on overall similarity with the assumption that each feature of an organism has equal taxonomic significance. Information is gathered concerning a large number of properties of each organism under study, the number of similar features between each pair of strains is counted and the strains are then sorted into groups whose members have a high percentage of similarities. The extensive computations involved in this method make the use of electronic computers almost mandatory.

In this study the methods of numerical taxonomy were applied to the classification of 70 strains of Pasteurella. Forty-five characters of each strain were tested. A selection of varied characters was chosen as listed in Table III.

METHODS AND MATERIALS:

Observation of Morphological and Cultural Characters

The nutrient broth used throughout was Oxoid #2. Nutrient agar was prepared by adding 2% agar (Difco) to nutrient broth and blood agar by adding 5% sheep's blood to nutrient agar.

The colonial morphology of all strains on blood agar was examined after 24 hours incubation at 37°C. The presence or absence of growth on nutrient agar and MacConkey's agar (Difco) after 24 hours incubation was noted. The character of growth in nutrient broth and in 5% serum broth was observed after incubation for 24 hours.

Table III

List of Differential Characters

Cultural behaviour

- Colony type on blood agar
- Production of hemolysis on blood agar
- Growth in simple media
- Growth on MacConkey's
- Type of growth in broth
- Iridescence on serum agar
- Strong fermentation

Morphological features

- Shape of bacillus
- Presence of capsule

Biochemical tests

- Indol production
- H_2S production
- Methyl red test
- Voges Proskauer test
- Gelatin liquefaction
- Nitrate reduction
- Production of catalase
- Production of oxidase
- Production of urease
- Staph. inhibition
- Utilization of citrate

Agglutination tests

- Agglutination with antiserum prepared from dog bite strain

Fermentative properties

Sugars -
- glucose
- sucrose
- galactose
- xylose
- maltose
- arabinose
- salicin
- lactose
- raffinose
- trehalose
- mannose
- rhamnose
- levulose
- dextrin

Alcohols -
- mannitol
- sorbitol
- adonitol
- inositol
- dulcitol
- glycerol

Antibiotic sensitivity

- penicillin
- streptomycin
- novobiocin

Pathogenicity tests

- mouse

Colonies grown for 24 hours on serum tryptose agar (Appendix 1) were examined by oblique, transmitted light for iridescence. A stereoscopic microscope was used for the examination. The light and microscope set up for obtaining the desired illumination is illustrated in Plate 2.

Microscopic morphology was observed in smears stained by Gram's method. Capsules were demonstrated by Jasmin's strain (43).

Fermentation of Carbohydrates and Polyalcohols

A method for performing fermentation tests was required which fulfilled the following criteria:

(1) the base medium must support adequate growth of all strains including those which were more fastidious. (The Pasteurella-like organisms and some of the Pasteurella multocida strains failed to grow in the usual peptone water base employed for fermentation tests).

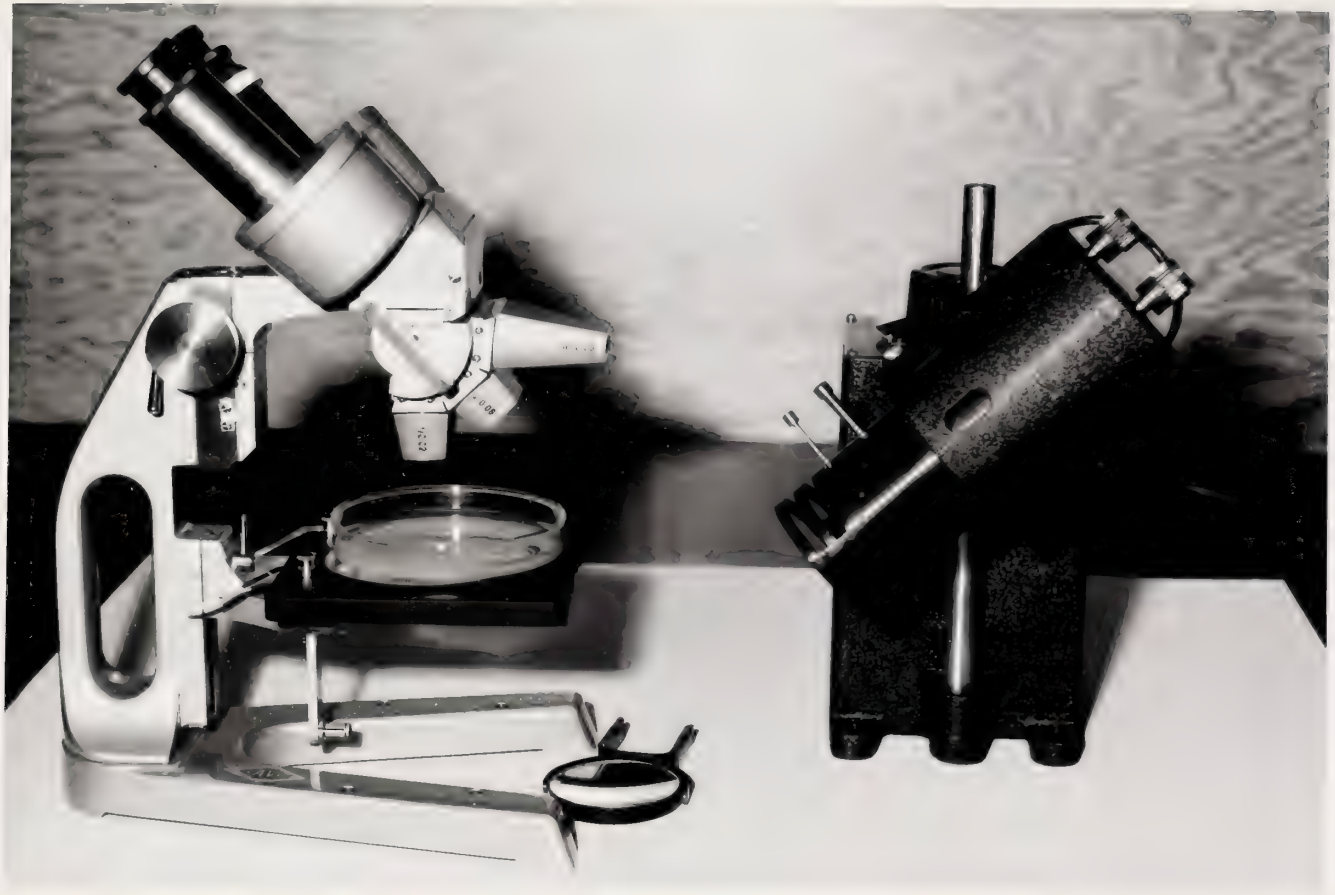
(2) the method must provide uniform and dependable results

(3) the method should avoid if possible the necessity of preparing many different kinds of media.

Cystine Trypticase Agar medium (BBL)* containing phenol red indicator (Appendix 1) proved to be a suitable basic medium for fermentation tests and supported good growth of all strains tested. The fermentable substance was added to the basic medium in the form of a sterile filter paper disc impregnated with the appropriate carbohydrate or polyalcohol (Taxos BBL). Fermentation of sucrose, glycerol and dextrin was tested in the same basic medium with 1% of the respective substrate added. All tests were observed for two weeks before being recorded as negative. To facilitate the reading

* Baltimore Biological Laboratory

PLATE 2



Microscope set-up for observing cultures by oblique transmitted light

of the sugar reactions a set of reading standards was prepared corresponding to known pH values. The uninoculated medium was pH 7.5 (0 on the reading scale). The degree of acidity was recorded as 1+, 2+ or 3+ corresponding roughly to pH values of 7.3, 7.0 and 6.6 or less. Those organisms which gave less than a 2+ reaction were classed as "weak fermenters".

Biochemical Tests

Indol production: Organisms were grown in an enriched peptone water medium (Appendix 1) for 48 hours and then tested with Kovacs'reagent.

Methyl-red and Voges Proskauer reactions: Organisms were grown in MR-VP medium (Difco) for 48 hours. Leifson's reagent was used for detection of acetylmethylcarbinol.

Hydrogen sulfide production and gelatin liquefaction were observed in the lead acetate-gelatin medium described by Talbot and Sneath (7). Tests were incubated for 14 days.

Nitrate reduction was tested according to the method outlined by Wilson and Miles (1).

Catalase production was tested by adding a few drops of 3% H_2O_2 to a 24 hour culture on serum tryptose agar slants and watching for evolution of bubbles.

Urease production was observed on Christensen's agar slants (44).

Citrate utilization was tested on Christensen's medium (45).

Oxidase activity was tested by the technique described by Kovacs in 1956 (46).

Staphylococcal inhibition: The presence of hyaluronic acid in capsules was demonstrated by a decrease in colony size of organisms growing near a streak of Staphylococcus aureus. The reaction is due to breakdown of hyaluronic acid by hyaluronidase elaborated by the staphylococci (47).

Other Tests

Antibiotic sensitivity: Tests were carried out on blood agar using Multidiscs (Colab.) The antibiotic concentrations employed were: penicillin 1 unit, streptomycin 5 mcgm, novobiocin 5 mcgm.

Mouse Pathogenicity: Mice were inoculated intraperitoneally with approximately 1 x 10^6 organisms. The test was recorded as positive when the mouse died within 48 hours and the organism was recovered from heart blood.

Agglutination: Antigens for agglutination tests were prepared by suspending live organisms in saline to correspond to the turbidity of Brown's tube #3 (Burroughs Wellcome). The antiserum used was one prepared against P.multocida strain #25 isolated from a dog bite infection. Tests were carried out in Widal tubes (10 x 75 mm) and read after 24 hours in a 37° waterbath.

RESULTS

The results of the tests are recorded in Table IV. On the basis of these tests a detailed description of human Pasteurella multocida can be documented.

Cultural Characteristics

Mucoid colonies were produced by 31 of 59 human strains after 24 hrs. incubation on blood agar. The degree of mucoidness ranged from cultures showing a confluent growth of large moist colonies to those showing smaller grey colonies with a tendency to run together. Other human strains formed, small discrete, translucent colonies about 1 mm in diameter. Growth on nutrient agar was poor and 4 strains failed entirely to grow on this

Table IV.

Results of Analysis of 70 Strains of Pasteurella

STRAIN NUMBER

CHARACTER	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Mucoid colony	-	-	+	-	+	-	-	+	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	+	+
Growth in simple media	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Iridescence	+	+	+	-	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	+
Broth turbid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coccobacilli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capsule	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	+
Staph. inhibition	-	-	+	-	+	-	-	+	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	-	-	+	+
Penicillin sens.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sens.	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-
Novobiocin sens.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Mouse pathogenicity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
Xylose	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcite	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Trehalose	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Levulose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	+	+	-	+	+	-	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	-	-	+	+	-	+
Dextrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Strong fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on MacConkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Agglutination	-	-	-	+	-	+	+	-	+	+	-	+	-	+	-	-	*	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	*	-	-
Haemolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CHARACTER	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
Mucoid colony	-	+	-	-	-	-	+	+	+	-	+	+	-	-	+	+	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+
Growth in simple media	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Iridescence	-	+	-	-	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	-	+	+	+	-	+	-	-	-	+	+	-	+	+	+
Broth turbid	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Coccobacilli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Capsule	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	+
Staph. inhibition	-	+	-	-	-	-	+	+	+	-	+	-	-	-	+	+	-	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-
Penicillin sens.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sens.	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+
Novobiocin sens.	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Mouse pathogenicity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Mannitol	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	-	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Sorbitol	-	+	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-	+	-	-	-	+	+	+	+	-	+	-	-	-	-
Xylose	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+	-	-	-
Maltose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Dulcite	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Trehalose	+	-	+	-	-	-	-	-	+	-	-	-	+	+	+	-	+	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Levulose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+
Glycerol	-	+	+	-	-	-	+	+	-	+	+	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-
Dextrin	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+
Indol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Strong fermentation	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Growth on MacConkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Agglutination	+	-	+	+	-	-	-	-	-	+	-	+	+	+	-	-	+	+	+	-	+	+	-	-	*	-	+	-	-	-	-	-	-	-	-
Haemolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-

* Strain autoagglutinated

medium. No growth occurred on MacConkey's agar. The iridescence of colonies on serum tryptose agar ranged from reddish yellow to bluish green to grey non-iridescent. Occasional cultures showed a mixture of blue-green iridescent and non-iridescent colonies.

Growth in broth appeared as uniform turbidity with a slight deposit at the bottom of the tube. Two strains gave a clear supernatant with a coarse deposit.

Morphology

Gram stained smears of colonies and of broth cultures of all strains showed a predominance of small coccal bacillary forms, with some longer rod-shaped organisms. Occasional filamentous bacilli were seen. Capsules were observed in 43 of the 59 strains. As expected the largest capsules were found on organisms which produced very mucoid colonies on culture. Typical bipolar staining bacilli were seen in Giemsa-stained smears of heart blood from mice infected with P. multocida. (Plate 1.)

Fermentation Reactions

Glucose, galactose, mannose and levulose were fermented within 48 hours with the production of acid. The majority of strains fermented sucrose, xylose, mannitol and sorbitol. Trehalose was frequently fermented as was glycerol after several days incubation. Occasional strains fermented arabinose, dulcite, maltose, lactose, raffinose and dextrin. Adonitol, inositol, salicin and rhamnose were not fermented.

Biochemical Reactions

All strains of P. multocida produced indol. Methyl red and Voges Proskauer tests were negative. The majority of strains produced small amounts of hydrogen sulfide in 2 to 7 days. Nitrates were reduced to nitrites.

Gelatin was not liquefied, citrate was not utilized. Catalase and oxidase were produced, urease was not produced. Colonies of very mucoid strains were of decreased size near a streak of Staphylococcus aureus .(Plate 3.) Other strains were unaffected by the Staphylococcus.

Other Reactions

All strains were sensitive to penicillin and the majority were also sensitive to streptomycin and novobiocin. Most strains were pathogenic to mice causing death within 24 hours of intraperitoneal injection. A few strains however produced no ill effects in mice.

COMPUTER ANALYSIS

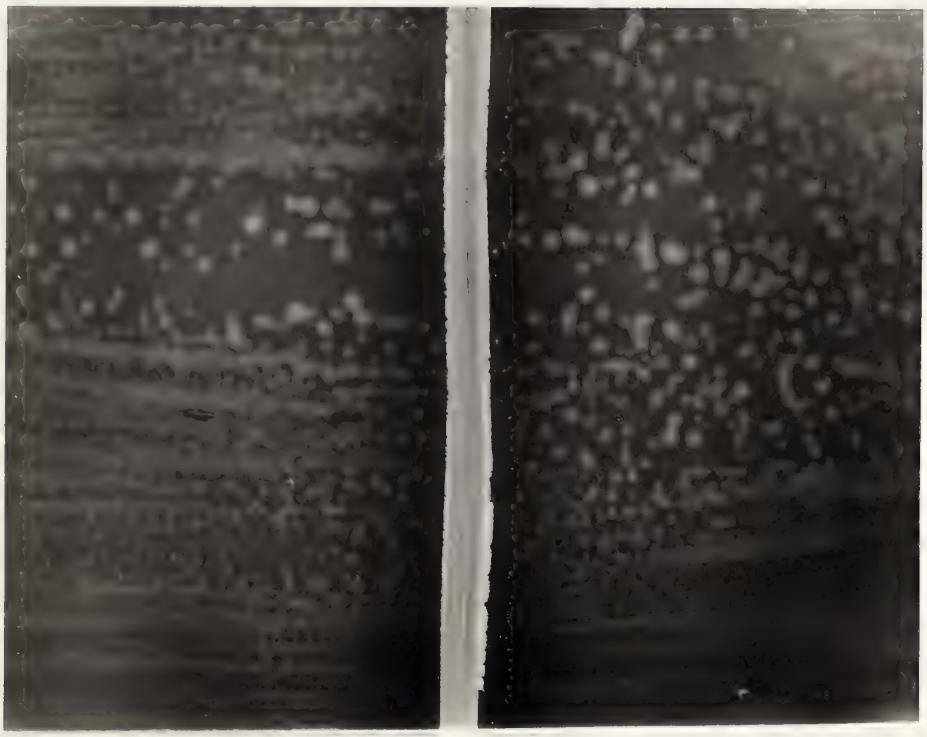
The results as recorded in Table IV were punched onto IBM cards. These data were then analysed by an IBM 1620 computer using a program devised by members of the University of Alberta Computing Center.

The first step was to determine the similarity coefficient (Sij) between each possible pair of strains. The simple matching coefficient described by Sokal and Sneath, 1963 (48) was used, that is the total matches (m) including both positive and negative matches between each pair of strains is divided by the total number (n) of characters compared

$$Sij = \frac{m}{n}$$

Thus from Table IV it is seen by comparing strains #1 and #2, that there are 44 matches among the 45 characters tested. The Sij value of strains 1 and 2 ($S_{1,2}$) is therefore 0.98.

PLATE 3



Staphylococcus effect - colonies of mucoid strain of P. multocida showing decreased size near a streak of Staph. aureus.

The similarity coefficients were delivered by the computer in the form of a 70 by 70 matrix, with the strains in the same order horizontally and vertically. For convenience this matrix was converted to a shaded diagram, part of which is shown in Figure 1. The squares are shaded to represent the degree of similarity between strains.

The next task for the computer was the re-arrangement of the strains so that very similar strains were brought together thus forming natural subgroups. Two methods of achieving this re-arrangement were used.

A. Linkage Method

In this method the computer first searched the data to pick out strains related at the highest possible level of similarity i.e. strains giving identical results in all 45 characters tested. The groupings formed were listed. Then the computer searched for strains related at the next highest level of similarity, the 0.98 level. New groups were formed and strains were added to existing groups. By testing at successively lower levels of similarity, groups were built up until eventually all strains united into one group. The criterion used for admission of a strain to an established group was that called the "complete linkage" criterion by Sokal and Sneath (48). That is, before a strain could join a group at for example, the 0.94 level, it must be related at that level or above with every existing member of the group. Using the data obtained from the computer a taxonomic tree or dendrogram was constructed. (Figure 2.)

Figure 1. Similarity Matrix Before Re-arrangement
(Only part of matrix illustrated)

Figure 2. Dendrogr[illegible]trains of Pasteurella

B. Weighted Method:

This was a more complex method in which the similarity matrix was examined as a whole. The object was to re-arrange the strains so that the higher similarity coefficients would lie close to the diagonal, thus making the most similar strains adjacent. To do this, each similarity coefficient (S_{ij}) was assigned a weight according to its position in the matrix. The weight given was equal to the square of the distance of the coefficient from the diagonal. For example, in the matrix below, the S_{ij} value circled is 3 positions off the diagonal, thus the assigned weight would be $3^2 = 9$

1.0			
.95	1.0		
.90	.85	1.0	
(.80)	.85	.90	1.0

The weighted sum of all Sij's was found. The computer then searched the data to find two strains which, when interchanged, would result in a decreased weighted sum. This was continued until the sum was the minimum obtainable. All necessary interchanges were made and the re-arranged similarity matrix was delivered by the computer. The re-arranged matrix is given in Figure 3 in the form of a shaded diagram.

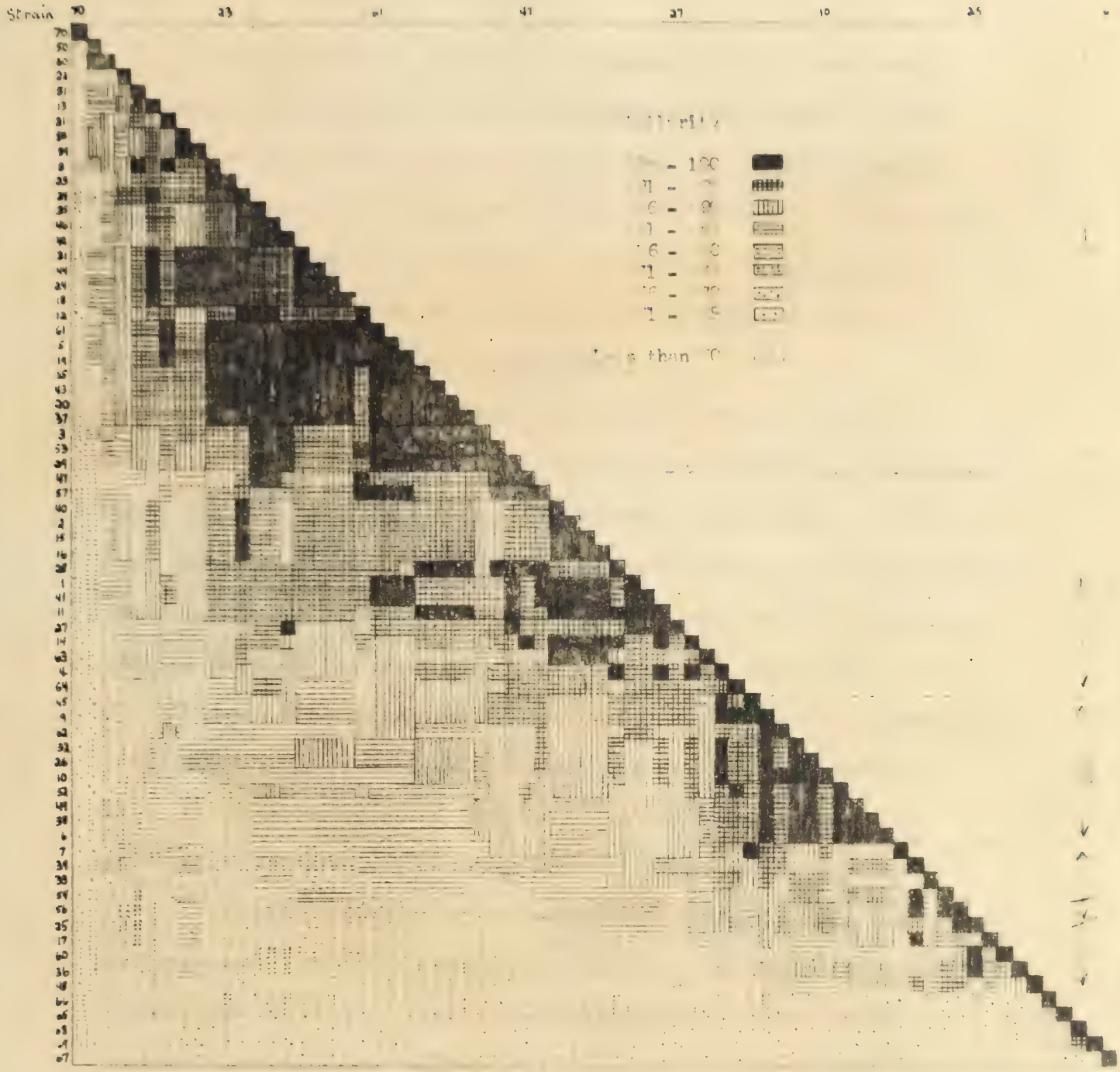


Figure 3. Similarity Matrix after Re-arrangement

RESULTS OF COMPUTER ANALYSIS

Both methods of analysis gave essentially the same results. Four groups or clusters were formed each containing strains that were closely similar. The strains of P. hemolytica, P. pseudotuberculosis and the Pasteurella-like organisms did not fall into any of these groups and showed a low degree of similarity with strains of P. multocida. However, a fairly high degree of similarity was shown between the Pasteurella-like strains and P. hemolytica.

The largest group, shown as Group I in Figures 2 and 3, consists almost entirely of strains isolated from respiratory infections. (Table V.) The one rabbit strain is included in this group. The second group, Group II, is made up of strains predominantly of respiratory origin but also includes strains from cerebrospinal fluid, ear and amputation site. Strains derived from animal bite wounds fall into Groups III and IV. Although the demarcation is by no means sharp there is a tendency to differentiate between strains originating from cats and those from dogs. All organisms derived from cat bites are found in Group III. Dog bite strains are found in both Groups III and IV.

The test results for the members of each group were examined to find the characters which tended to differentiate one particular group of strains from the others. Only strains common to a group by both the "linkage" and "weighted" methods of cluster analysis were considered. There were 24 such strains in Group I, 11 in Group II, 12 in Group III and 8 in Group IV.

TABLE V.

Grouping by Numerical Taxonomy vs. Source of Strain

Source	Group by Numerical Taxonomy			
	I.	II.	III.	IV.
Respiratory tract	25	9	1	0
C.S.F.	0	2	0	0
Peritoneum	0	0	1	1
Ear	1	1	0	0
Wounds & abscesses	0	1	1	2
Dog bite	0	0	4	4
Cat bite	0	0	5	0
No information	1	0	0	0
Rabbit	1	0	0	0
Dog	0	0	1	1
Cat	0	0	1	0
Buffalo	0	1	0	0

Of the 45 characters tested, 14 were found to give results that varied signifcantly from group to group. The frequency with which the character was found in each of the groups is indicated in Table VI. Strains of Group I were those forming mucoid colonies due to the presence of large amounts of hyaluronic acid in their capsules. Strains of Group II were capsulated but did not produce a mucoid growth in culture. Failure to ferment trehalose was a constant characteristic of these strains. Non-capsulated strains fell into Groups III and IV. The incidence of glycerol-fermenting strains was high in Group III while members of Group IV were characterized by lack of the ability to ferment mannitol, sorbitol and glycerol. The only maltose-fermenting strains of P. multocida belonged to Group IV.

TABLE VI.

Frequency of Certain Characters among Sub-groups of *Pasteurella multocida*

Character	Group I	Group II	Group III	Group IV.
Colony mucoid	1.0	0.09	-*	-
Iridescence	1.0	0.91	-	-
Capsule	1.0	1.0	0.25	-
Staph. inhibition	1.0	-	-	-
Streptomycin sensitivity	0.79	0.45	0.92	1.0
Mannitol fermentation	0.92	1.0	1.0	-
Sorbitol "	0.88	1.0	0.75	-
Xylose "	0.96	1.0	0.75	0.38
Maltose "	-	-	-	0.38
Trehalose "	0.29	-	0.50	0.38
Glycerol "	0.38	0.36	0.92	-
Strong fermentation	1.0	1.0	1.0	0.38
Agglutination with dog bite strain antiserum	0.08	0.18	1.0	0.75

* - indicates0 frequency

DISCUSSION

It is beyond the scope of this dissertation to attack or defend the philosophy of numerical taxonomy. The theoretical basis of this method has been discussed thoroughly in the publications of Sneath and Sokal over the past 7 years, and although taxonomists may disagree on the applications and practicality of numerical taxonomy, it is difficult to dispute its logic. What must be considered here is whether the procedures used in this investigation allow fulfilment of the concepts of numerical taxonomy.

A basic axiom of numerical taxonomy is that the classification should be based on as many characters as possible. Obviously there are practical limits to the number of characters that can be determined. It is often difficult to find a large number of characters that will vary among strains of bacteria especially when attempting to classify within a species as in this study, and there is no value in including characters which do not vary within the sample of organisms; Sneath, 1962 (49) recommends that at least 40 to 50 characters should be determined and preferably several hundred. The number in this investigation, 45, is probably just on the borderline of acceptability but it can be argued that once a certain number of characters have been recorded, the inclusion of additional characters provides little gain in information.

The other criteria of a classification based on the methods of numerical taxonomy were, I believe, satisfied. The characters tested were varied, representing morphological, physiological, fermentative and nutritional features of the organisms. All characters were given equal weight in determining taxonomic relationships. The methods of clustering, although somewhat different from those used by Sokal and Sneath, are, I believe, quite valid. The fact that remarkably similar results were given

by both methods of clustering used in this study supports their validity. Discussion of the taxonomic relationships revealed by numerical methods will be deferred until after consideration of classification by serological techniques.

Part IV. Classification of Pasteurella Strains by Serological Methods.

INTRODUCTION

The variety of serological techniques which have been used in the attempted classification of Pasteurella multocida reflects the inherent problems involved in a serological classification of this species. Several of these techniques were investigated in a series of preliminary experiments and the drawbacks of each were soon appreciated. Capsulated strains proved to be inagglutinable in homologous antisera, thus simple agglutination tests were unsuitable for classification of the majority of the strains of this series. Organisms which were non-virulent or of low virulence could not be typed by cross protection tests in mice. Complement fixation methods revealed a confusing degree of cross reactivity among strains. Precipitation and capsular swelling tests were unsatisfactory.

However, two serological methods warranted further investigation, the indirect hemagglutination test and the use of fluorescent antibody techniques. The first of these has been successfully applied by Carter (21) to the classification of several hundred strains of Pasteurella multocida mainly of animal origin. Some human strains have also been typed by Dr.Carter including about 50 strains of this series.

The fluorescent antibody technique on the other hand has not previously been used to classify strains of P. multocida although Bain (24) has noted its promise as a means of demonstrating special antigens of Pasteurella.

HEMAGGLUTINATION TEST

Classification of P. multocida by means of an indirect hemagglutination test was introduced by Carter in 1955, (19). A crude extract containing soluble antigen is prepared by heating a suspension of organisms at 56°C. The antigen is adsorbed onto the surface of erythrocytes which will then agglutinate in the presence of specific antisera.

The hemagglutination (HA) technique used in this study was essentially that described by Carter. Following initial experiments using the Carter method, some modifications were introduced into the procedure. The modified procedure is described below.

Methods and Materials

Red blood cells

Type O human red blood cells obtained from the local Red Cross Blood Transfusion Service were used throughout. The cells were maintained in the original acid-citrate-dextrose (ACD) solution and could be used satisfactorily for up to two weeks.

For each day's tests, the required volume of cells was removed and washed three times in buffered saline.

Saline solution

Phosphate buffered saline (pH 7.2) was substituted for the normal saline of the Carter method.

Preparation of extract

Growth from an 18 hour culture on a blood agar plate was washed off with 5 ml buffered saline. The suspension of organisms was placed in a 56°C waterbath for 30 minutes and then centrifuged at 5000 RPM for 20 minutes. The resultant extract (the supernatant) was water clear.

Sensitization of red blood cells.

To each extract was added 0.2ml of packed, washed, red blood cells. The cell suspension was mixed and placed in a 37°C incubator for 2 hours. The sensitized cells were then washed three times in buffered saline and made up to a 1% suspension.

Preparation of antisera

Strains used for preparation of antisera were:

Type B serum - Strain #63 - Type B strain from Ontario Veterinary College

Type D serum - Strain #15 - isolated from human sputum - identified as Type D by Carter

Type A serum - Strain #37 - isolated from human sputum - identified as Type A by Carter

(i) Types B and D antisera

Growth from 24 hour cultures on tryptose agar was washed off with normal saline, centrifuged, the supernate removed and replaced with sufficient 0.1% formol saline to give a suspension corresponding to Brown's opacity tube #3 (Burroughs Wellcome). The suspension was incubated at 37°C for 24 hours and then checked to ensure sterility.

> The inoculation schedule was as follows:- The rabbits received an initial subcutaneous injection of 1 ml of the above suspension followed by a series of 9 intravenous injections given twice weekly. The dose size was gradually increased from 1 ml to 5 ml with a total of 25 ml of antigen being given over the injection schedule. If test bleedings 1 week following the last injection failed to show a sufficient level of antibody, further subcutaneous injections of live organisms were given until a satisfactory titer was achieved.

(ii) Type A antisera

Difficulty was encountered in preparation of adequate type A antisera. The above method using either heavily capsulated or moderately capsulated strains failed to stimulate sufficient antibody production. It was considered that the presence of large amounts of

hyaluronic acid in the capsules of type A strains might possibly have an inhibitory effect on antibody production. Accordingly injections of hyaluronidase treated organisms were given to rabbits but still with unsatisfactory results.

The use of organisms incorporated in adjuvant was then tried and proved to be more successful. A suspension of organisms was prepared in a similar manner to that described in section (i) above, but was made up to the turbidity of Brown's tube #6. Equal proportions of the antigen and of Freund's complete adjuvant (Difco) were mixed by shaking for 20 minutes on a Kahn shaker. Rabbits were injected with a total of 1 ml given in a divided dose, each week for a period of 3 weeks. The material was injected into the muscle of the back of the neck. The rabbits were bled out 1 month following the last injection.

Performance of test

Initially the tests were carried out in 12 x 100 mm tubes but it was found that a modification of the procedure using perspex agglutination plates* offered several distinct advantages. Less antiserum was required in the plate method and the results could be read sooner and more easily. The procedure adopted was as follows:

Doubling dilutions of antiserum (1/10 - 1/2560) were prepared in buffered saline. A volume of 0.2 ml of each dilution was transferred to the appropriate well in the agglutination plate. Then, an equal volume of the 1% suspension of sensitized red blood cells was added to each serum dilution. To ensure adequate mixing, the plate was agitated for about 20 seconds on a flat surface in a combination of rotary and back and forth movements. The plate was covered with a piece of glass and left at room temperature for approximately 1 hour to allow the red cells to settle. Readings were taken at that time and again following overnight refrigeration.

A control consisting of 0.2ml of sensitized cells and 0.2ml of saline was included for each antigen tested. Control tests were also carried out on each new lot of antisera. A 1/10 dilution of the antiserum was tested with a 1% suspension of normal erythrocytes to rule out the possibility of non-specific agglutination.

Sera were inactivated at 56°C for 20 minutes before use in hemagglutination tests.

* World Health Organization agglutination plates - 80 hole 1.5 ml capacity

Pattern of Hemagglutination

Two different patterns of hemagglutination were noted. In the first the red cells settled to form a button whether the reaction was positive or negative. Hemagglutination only became evident upon gentle shaking of the agglutination plate. In a negative reaction the red cells resuspended quickly and uniformly, in positives clumping was observed. This type of reaction is illustrated in plate #4. It was a characteristic of tests employing rabbit sera.

The second type of pattern was similar to that found in virus hemagglutination tests, the cells in a positive reaction settling as a shield or carpet with the negatives forming a button (plate #5). This pattern was observed with some positive human sera.

Results of Hemagglutination Tests

The results of the tests are summarized in tables VII - IX.

Strains 1 - 55 had previously been typed by Dr. G. R. Carter*. Twenty of these strains plus strains 56 - 64 were tested by the writer.

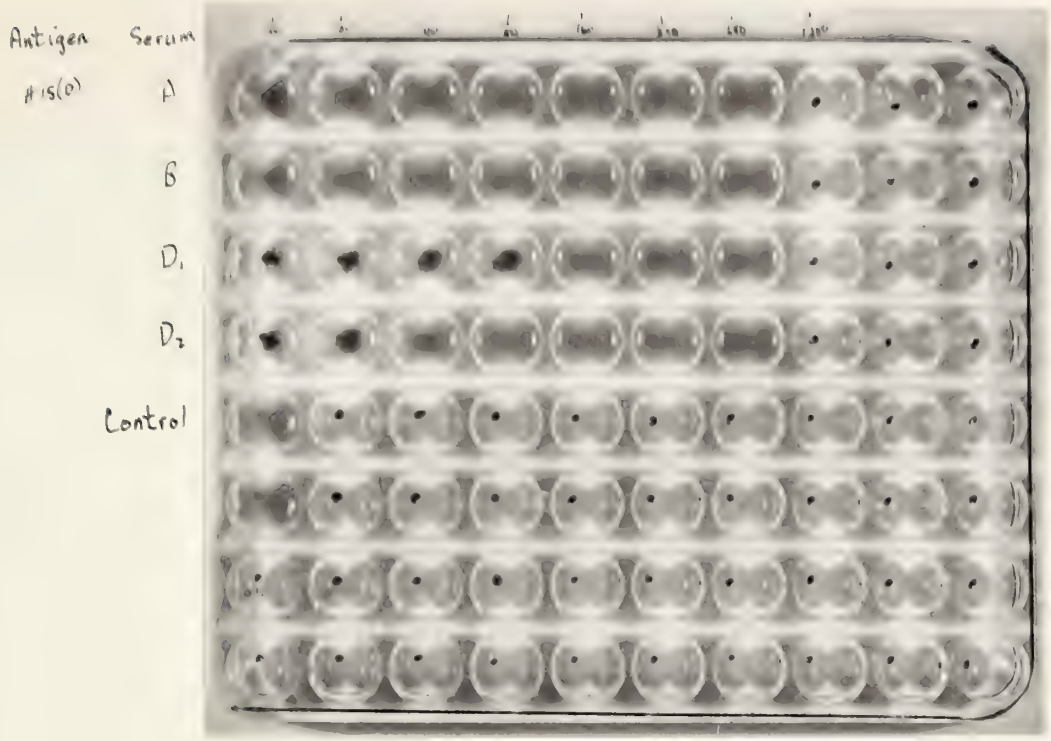
From table VII it is noted that only capsulated strains were typeable by the HA test. All except one of the mucoid strains belonged to type A.

A breakdown of the typing results according to the source of the strains is shown in table VIII. Organisms from respiratory tract, C.S.F. and ear were typeable and belonged to types A or D. Without exception strains isolated from dog or cat bite infections could not be typed.

In table IX the serological classification by means of the HA test is compared to groupings obtained by the taxonomic methods of part III. Type A strains correspond closely to group I of the computer grouping, type D to group II, and the non-typeable strains fell into groups III and IV.

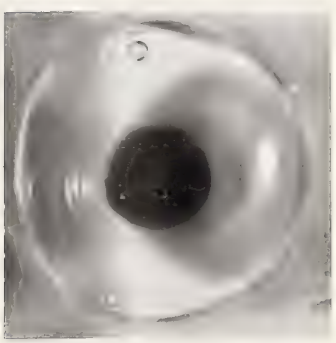
* formerly of Animal Diseases Research Institute, Hull, Quebec.

PLATE 4

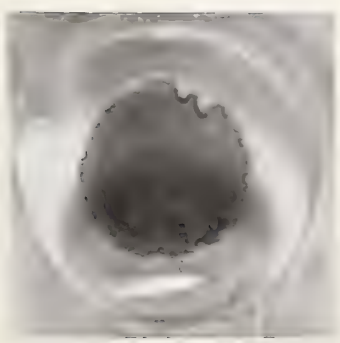


Hemagglutination pattern 1. Agglutination of red cells evident after gentle shaking of plate.

PLATE 5



Negative reading



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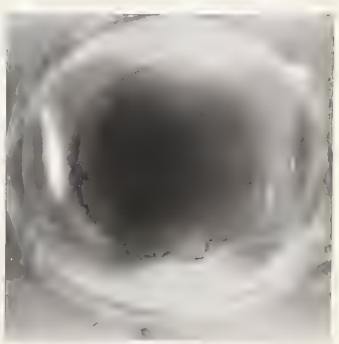
Positive readings



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Hemagglutination pattern 2. Red cells settle in "shield" pattern.

TABLE VII.

Results of Hemagglutination Tests with P.multocida Strains

	Hemagglutination type				
	A.	B.	D.	NT*	TOTAL
All strains	29	1	9	25	64
Capsulated strains	29	1	9	5	44
Non-capsulated strains	0	0	0	20	20
Mucoid strains	29	0	1	0	30

TABLE VIII.

Comparison of Hemagglutination Type and Source of Strain

Source	Type			
	A.	B.	D.	NT*
Respiratory tract	25	0	7	3
C.S.F.	0	0	2	0
Peritoneum	0	0	0	2
Ear	2	0	0	0
Wounds & abscesses	0	0	0	4
Dog bite	0	0	0	8
Cat bite	0	0	0	5
No information	1	0	0	0
Non-human strains	1	1	0	3

* Non-typeable

TABLE IX

Comparison of Hemagglutination Type and Grouping by Numerical Taxonomy

Group by Numerical Taxonomy	Hemagglutination Type			
	A.	B.	D.	NT
I	27	0	0	1
II	2	1	9	2
III	0	0	0	14
IV	0	0	0	8

Evaluation of the Hemagglutination Test

A number of difficulties were encountered in carrying out hemagglutination tests. A major problem has already been mentioned - the preparation of high titer antisera particularly for type A Pasteurella multocida. The use of organisms incorporated in adjuvant as the antigenic stimulus was a partial answer but even then the maximum titer of the antiserum obtained was only 1/640. It may be that a better response could be achieved by employing partially purified preparations of the type specific antigen incorporated in adjuvant. The poor response to the type specific antigen appears to be because of the weak nature of the antigen rather than due to an inhibitory effect of hyaluronic acid on antibody production. Hyaluronidase treated organisms failed to produce an adequate antibody response. Furthermore, immunization of a rabbit with a capsulated type A strain produced no significant level of hemagglutinating antibody but did produce a high level of agglutinating antibody to decapsulated type A organisms indicating that the antibody response to other antigens of P. multocida is not inhibited by the presence of hyaluronic acid in the capsule.

Hemagglutination tests involving type A strains varied considerably in the degree of reaction produced. Some strains gave a very weak reaction positive only in a 1/20 dilution of antiserum, others gave a stronger reaction. Even duplicate tests with the same strain showed considerable variation. The amount of sensitizing antigen in the crude extracts as prepared by the Carter method is unknown and will certainly differ markedly from one preparation to the next. It has been shown by Bokkenheuser and Koornhof, 1959 (50) that the titer of a given serum will vary according to the quantity of antigen adsorbed onto the red cells. This could well explain the variations found

in this investigation. The only method of overcoming this unknown factor is by preparing a purified lipopolysaccharide extract of the organism to be tested and then exposing the red cells to the optimum amount of this antigen. Such a procedure is recommended by Landy, 1954 (51) but obviously is not suited as a routine procedure when large numbers of organisms have to be typed. Even without this added step the indirect hemagglutination test is a time consuming and somewhat tedious test. Because the longest and most critical step in the procedure is the preparation of the sensitized cells, HA tests are more readily applicable to detection of antibody rather than to identification of an antigen. In part V of this investigation, hemagglutination methods are applied for this purpose - detection of antibody in human sera - and prove to be a useful and sensitive tool.

The specificity of the hemagglutination test appears to be of a high order. There was no instance of cross reaction; typeable strains of P. multocida reacted with only one of the type specific antisera available, strains of other species of Pasteurella did not react with multocida sera in the hemagglutination test.

The value of HA tests as a method of classification of human strains of P. multocida will be discussed fully in part VI. It may be noted here, however, that almost 40% of the multocida strains investigated in this series could not be typed by hemagglutination methods.

FLUORESCENT ANTIBODY TECHNIQUE

Within recent years the fluorescent antibody technique has gained widespread acceptance as a useful tool for detection of antigen or antibody. The method has proven to be very sensitive and can readily be applied to many types of antigen-antibody systems. It seemed likely that the use of a fluorescent antibody (FA) technique in classification of P. multocida might overcome some of the difficulties encountered in other serological methods. The hyaluronic acid of the capsule which inhibits ordinary agglutination and interferes to some extent with hemagglutination should have no adverse affect in a FA technique. Once specific antisera are prepared, the method is relatively easy to carry out and is suitable for testing a large series of strains.

In this study an indirect method was used. The fluorescing substance was fluorescein-labelled anti-rabbit globulin. The method is that given in the U.S. Public Health publication "Fluorescent Antibody Techniques" (52) and is described below.

Methods and Materials

Pasteurella multocida antisera: The antisera used were those prepared for the hemagglutination tests (see page 46).

Labelled anti-globulin: BBL Fluorescein-labelled anti-rabbit globulin lot #8-2960 was used.

Microscope: A standard Zeiss microscope equipped with a darkfield condenser was used.

Light source: The source of ultra-violet light was an Osram HBO-200 high pressure mercury lamp.

Filters: The following filters were used:

Heat stopping filter	-	KG1
Primary exciter filter	-	BG 12
Barrier filters	-	Zeiss 47 and 50

Performance of Indirect Test

A suspension of the organisms to be tested was prepared by mixing one loopful of a 24 hour growth from blood agar in distilled water. The turbidity of the bacterial suspension was roughly equivalent to Brown's tube #3. Smears were prepared on new glass slides that had been cleaned in acetone. One loopful of the suspension was used for each smear. Smears were allowed to air dry then fixed by passing quickly through a gas flame.

Smears were covered with one drop of the appropriate P. multocida antiserum and placed at room temperature for 30 minutes, in a moist atmosphere (provided by covering the slides with a petri dish lid lined with moistened filter paper).

The slides were then rinsed in phosphate buffered saline (pH 7.2) by first allowing the saline to flow gently over the slide thus removing most of the antiserum, then flooding the slide with buffered saline and leaving for 10 minutes. Fresh saline solution was added two or three times during the 10 minute period. The slides were then rinsed briefly in distilled water and gently blotted with bibulous paper.

The smears were covered with one drop of the labelled anti-globulin and again placed in a moist atmosphere for 30 minutes followed by rinsing

in buffered saline and distilled water as above. The slides were drained and blotted dry.

Smears were mounted by placing one drop of glycerol saline (9 parts glycerol and 1 part buffered saline) on each smear and covering with an acetone cleaned cover slip. The preparation was sealed with melted paraffin.

All smears were examined under the microscope using ultra-violet illumination, a darkfield condenser and an oil immersion objective.

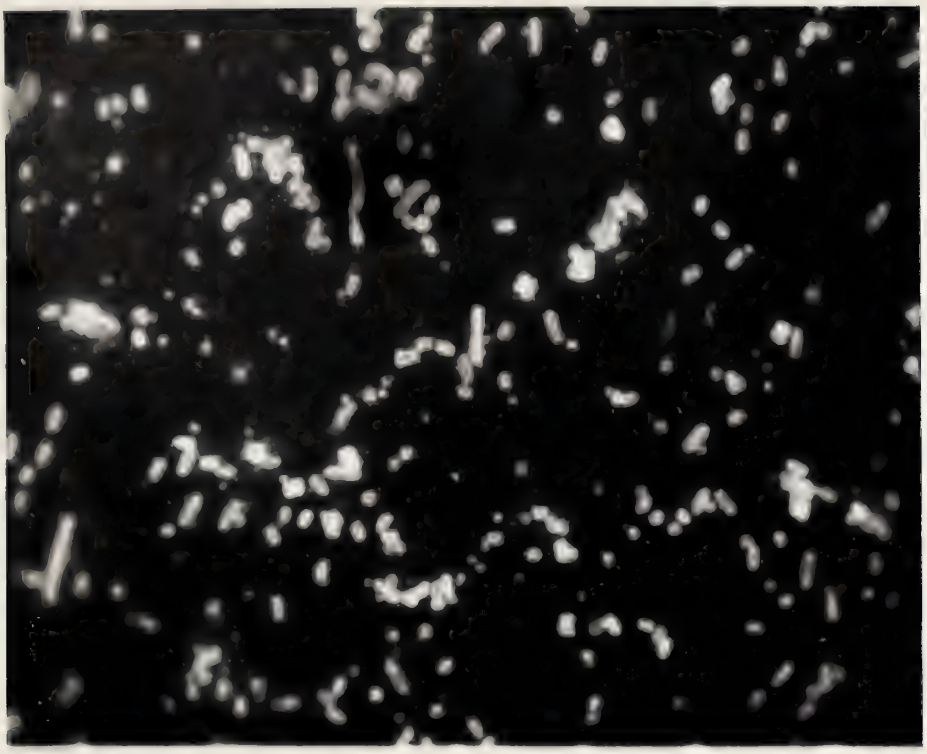
Appearance of Smears

In positive smears a bright fluorescence was observed. The fluorescent staining bacilli showed up as bright outlines against a dark background. The smears were rated as +, ++ or +++ depending on the degree of fluorescence. Photographs of positive smears are shown in plates 6 and 7. In negative smears the organisms could be seen but were dull and non-fluorescent.

Titration of Reagents:

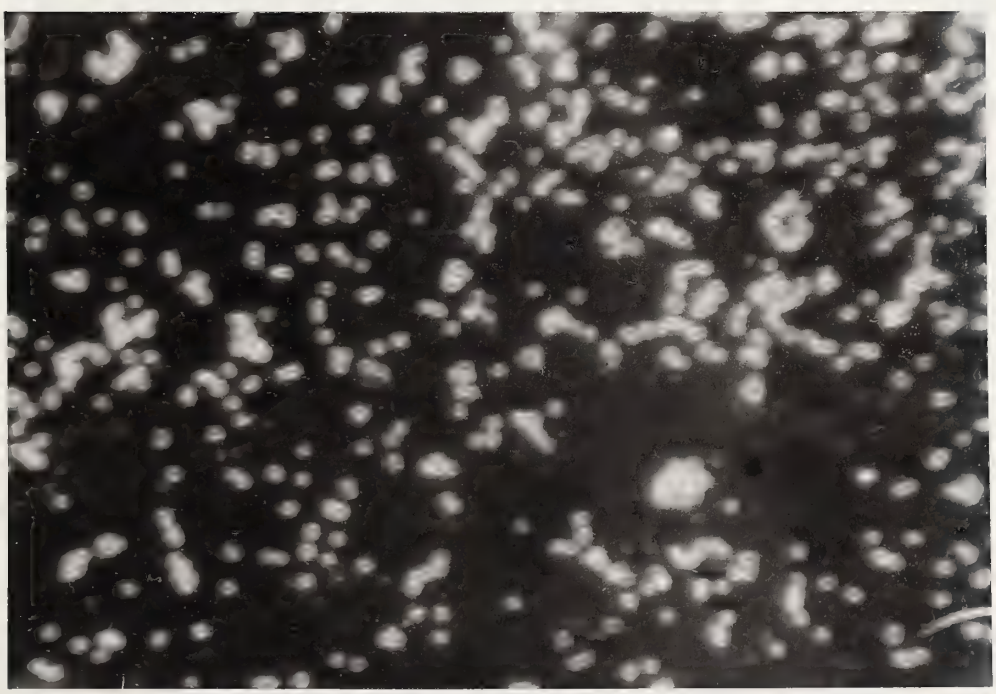
Checkerboard titrations were carried out to determine the optimum concentrations of P. multocida antisera and labelled anti-globulin to use in the FA test. Multocida serum was diluted in buffered saline, the anti-globulin in 2.5% bovine albumin. Smears were made of a known type of P. multocida. The appropriate dilutions of multocida antiserum and anti-globulin were added according to the technique just described. The results of a typical titration are presented in table X.

PLATE 6



Fluorescent-antibody staining of P. multocida by indirect method. 2000X

PLATE 7



F.A. staining of P. multocida. Note peripheral staining of organisms. 2000X

TABLE X.

Titrations of Reagents for Indirect FA test

Anti* P.multocida serum / Labelled anti-rabbit globulin	1/1	1/5	1/10	1/20
1/1	++	++	++	+
1/2.5	+	++	+++	++
1/5	+	++	++	+++**
1/10	+	+	++	++

Antigen: P.multocida strain #37 (type A)

*Antiserum: A 37 - prepared in rabbits against type A strain #37

**Optimum proportions: P.multocida antisera 1/20 Labelled anti-globulin 1/5

Note: Smears in which undiluted or low dilution of anti-globulin were used showed a dull fluorescent background. With higher dilutions of anti-globulin the background was dark thus giving a better contrast for fluorescing organisms.

Absorption of Antisera

P. multocida antisera which proved to be type specific in hemagglutination tests showed a high degree of cross reaction when employed in FA procedures. Type A, type D and non-typeable strains all reacted with both types A and D antisera. In an attempt to eliminate this cross reaction serum absorptions were carried out.

Type A antisera: Type A serum was first absorbed with a non-capsulated strain of P. multocida (strain #32) in order to remove common O antigens. The absorption procedure was as follows:

> The organism to be used for absorption was inoculated to nutrient broth and incubated for approximately 5 hour in a 37°C waterbath-shaker. Twelve nutrient agar plates containing 40 ml agar/plate were each inoculated with 0.3 ml of the broth culture, spread with a glass spreader and incubated for 18 hrs. Growth from the plates was washed off with 0.5% saline and heated at 100°C for 1 hour. The organisms were centrifuged, washed once in 0.5% NaCl, re-centrifuged and the supernatant discarded.
>
> One milliliter of the serum to be absorbed was diluted in 9 ml of 0.5% phenolized saline and added to 1/2 of the packed cells. The mixture was shaken, placed in a 37°C waterbath for 4 hours, then centrifuged and the supernatant added to the second portion of packed cells. The mixture was again incubated at 37°C for 2 hours and then placed in the refrigerator overnight. The organisms were removed from the absorbed serum by centrifugation.

The absorbed serum was then checked in FA tests. Cross reaction still occurred with type D strains so further absorption was necessary. Type D strain #15 was used for the second absorption.

The results of FA tests with representative A, D and non-typeable (NT) strains and type A serum before and after absorption are presented in table XI.

Table XI

Absorption of Type A antisera

Strain #	Type	TypeA serum unabsorbed	Type A serum absorbed with	
			#32 (NT)	#32(NT) and #15 (D)
#37	A	+++	+++	+++
#15	D	+++	++	-
#32	NT	+++	-	-

To determine the specificity of the absorbed serum it was now tested with other P. multocida strains of known hemagglutination type. The results are presented in the left hand column of table XII. It was found that all type A strains tested reacted with the absorbed A serum but in addition, 2 of 5 type D strains, and 1 of 5 non-typeable strains gave positive results. A further absorption was necessary to remove this cross reaction, the absorbing strain used was type D strain #1.

After this final absorption the serum proved to be specific for type A strains of P. multocida - see table XII - right hand column. The serum did not react with P. hemolytica or P. pseudotuberculosis.

Table XII

Further F.A. Tests with Absorbed Type A Antisera

Test organisms	Type A serum absorbed with	
	#32 (NT) and #15(D)	#32 (NT), #15 (D) and #1 (D)
Known Type A strains		
#37	+++	+++
#34	+++	+++
#42	+++	+++
#55	+++	+++
Known Type D strains		
#15	-	-
#16	-	-
#40	-	-
#1	+++	-
#41	+++	-
Known NT strains		
#32	-	-
#33	-	-
#49	-	-
#38	-	-
#52	+++	-

Type D antisera: Similarly attempts were made to prepare a specific type D antiserum. The serum was first absorbed with a non-typeable strain of P. multocida #49. Cross reaction occurred with the serum and type A strains, so a second absorption was carried out with A strain #37 as the absorbing organism. A summary of FA tests using the serum before and after absorption is presented in table XIII.

Table XIII

F.A. Tests with Type D antisera

	Type D serum unabsorbed	Type D serum absorbed with #49 (NT)	Type D serum absorbed with #49 (NT)and #37(A)
Known A strains			
#37	+++	+++	-
#34	nd	nd	-
#42	nd	nd	+++
Known D strains			
#15	+++	+++	+++
#16	nd	+++	++
#1	nd	+++	+++
#41	nd	+++	+++
Known NT strains			
#49	+++	-	-
#52	nd	nd	-

nd = not done

Evaluation

Although more work must be done before a complete evaluation can be made, the findings to date indicate that P. multocida can be classified by means of a fluorescent antibody technique. It is apparent from this study that careful absorption of antisera is required before the test can be used to identify specific types. Further work is necessary to establish the specificity of the reaction. The absorbed sera that were prepared in this investigation did not react with P. hemolytica, P. pseudotuberculosis or the Pasteurella-like organisms, and the absorbed type A serum, as far as these tests revealed, reacted only with type A strains of P. multocida. The D serum however, after two absorptions, still reacted with one of the type A strains but a further absorption of the serum likely would remove this cross reaction.

In this investigation the antisera were absorbed so as to reveal the type specific capsular antigens of Pasteurella multocida. But the sera might equally as well have been absorbed to reveal other antigens of this species. For example, it may be that certain somatic antigens will prove to be of value in subdividing the present types. Antisera could probably be prepared, by careful absorption to reveal such antigens in a FA procedure. It is apparent from the absorptions carried out in this investigation that sub-types do exist in both type A and type D. These sub-types might well prove significant in classification of P. multocida.

FA tests are relatively simple to carry out. They are sensitive, rapid and readily adapted for testing any number of organisms in one series of tests. Adequate controls must, of course, be included. Each serum must be tested with known positive and negative strains.

Part V. Immunological Response to *P. multocida* Infections in Humans.

SEROLOGICAL TESTS ON PATIENTS WITH KNOWN *P. multocida* INFECTION

Methods

Antibody studies were carried out on sera from 6 patients with known *P. multocida* infection. Five of the patients had infection of the respiratory tract, one had an infected cat bite. Each serum was tested in agglutination and hemagglutination tests with the strain recovered from the patient and with other representative strains of *P. multocida*.

The procedure for agglutination tests was that described on page 28. Antigens consisted of saline suspensions of living organisms. Hemagglutination tests were carried out as described on page 48. Six serum specimens submitted to the Provincial Laboratory for routine syphilis serology were included as controls.

Results

The results of the tests are summarized in table XIV. Agglutination and hemagglutination tests with the six control sera were negative.

P. multocida was isolated from patient F.L. on two separate occasions. The first isolation (strain #12) was in November 1958, the second (strain #57) in October 1962. The serum sample was obtained at the time of the second isolation.

High levels of antibody were found in 4 of the 5 patients with respiratory infection, a low level in the one patient with cat bite infection.

Table XIV Serological Tests on Patients with Known P.multocida Infection

Patient	Diagnosis	Strain* No.	Type	Agglutination Test		Hemagglutination Tests		
				Patient's strain	NT strain #52	Patient's strain	Type A strain #37	Type D strain #15
R.H.	cat bite	26	NT	1/40	Neg.	not done	Neg.	not done
G.F.	chronic bronchitis	42	A	neg.	Neg.	Neg.	Neg.	Neg.
A.M.	chronic bronchitis	45	NT	1/1280	1/160	Neg.	1/80	Neg.
F.L.***	bronchiectasis	12	A	**var.a - Neg. var.b - 1/2560	1/320	not done	1/2560	Neg
		57	NT	1/2560		Neg.		
H.W.	bronchiectasis	46	A	Neg.	1/160	1/40	1/80	Neg.
E.S.	U.R.I.	58	A	Neg.	Neg.	1/160	1/320	Neg.

* Refers to strain number in table 1.
** Two variants found in strain #12: variant a, produced iridescent colonies, variant b, non iridescent colonies
*** Serum obtained at time of 2nd isolation

Discussion:

The results show that comparatively high titers of antibody may be produced in patients with respiratory infection with P. multocida. Of 5 such patients investigated, antibody to type specific capsular substance was demonstrable in 4, antibody to somatic substance in 3. The former was revealed by hemagglutination tests, the latter in agglutination tests in which non-capsulated organisms were employed as the antigenic suspension.

Capsulated strains of P. multocida proved to be inagglutinable as illustrated by the results with patient F.L. A capsulated strain isolated from F.L. was not agglutinated by her serum, but a non-capsulated variant was agglutinated to high titer. This is a well recognized finding with P. multocida but is overlooked by practically all workers who have carried out serological investigations of patients with P. multocida infection. For example, in a patient with P. multocida in chronic nasal sinusitis of 12 years duration described by Bartley in 1960 (53) agglutination tests with the patient's organism and serum in 1947 were negative, in 1959 a titer of 1/40 was found. Similarly Brodie and Henderson in 1963)54) record a case of bronchiectasis of 4 years duration in which an agglutination titer of only 1/32 was obtained. In an attempt to increase the patient's titer, a vaccine prepared from the patient's strain was administered. After 12 injections, the antibody titer was 1/64, after 16 injections the titer was zero. In both these cases, serological investigation by means of the hemagglutination test would very likely have demonstrated a high titer of antibody.

The strain isolated from patient A.M. in this investigation could not be typed by the hemagglutination test due to absence of type specific capsular substance. The presence of type A antibodies in A.M's serum suggests that the strain was originally a type A strain but had now

lost its type specific capsular antigen. A comparable finding is shown with patient F.L. The strain isolated in 1958 was typeable although non-capsulated variants were observed in cultures. The strain isolated in 1962, on the other hand, could not be typed, due to lack of type specific capsular antigen. Serum obtained at the time of the second isolation, however, showed a very high titer of type A antibodies. Carter (1962) observed that non-typeable strains are frequently obtained from chronic processes in fowl and also in carrier states.

SCREENING OF SERA FROM PERSONS NOT KNOWN TO HAVE P.multocida INFECTION

The evidence just presented suggests that respiratory infection with P. multocida is generally associated with the presence of specific antibodies in the patient's serum. Thus a serological test for antibodies to P. multocida can be employed to indicate past or present infection with the organism. The serological method best suited for this purpose is, I believe, the hemagglutination test using red cells sensitized with type specific antigen. The specificity of this technique has been demonstrated in part IV of this investigation.

The HA method was used to screen samples of sera from persons not known to have P. multocida infection in order to gain some idea of the probable incidence of antibodies in the general population. The antigen was prepared from a type A strain (the type most often found in respiratory infection).

Methods

Between January 28th and May 10th, 1963, 1000 samples of human sera submitted to the Provincial Laboratory for routine serological tests for syphilis were screened for antibodies to type A P. multocida. Tests were confined to sera from patients over 30 years of age who had been admitted to the Mewburn Pavilion or the medical wards of the University Hospital in Edmonton. By limiting the tests to this group it was felt that the likelihood of finding "positives" was increased. (Undue pessimism was entertained at the beginning of the survey about the chances of finding positive sera). All sera were inactivated at 56°C before testing.

Screening Test

The procedure for the hemagglutination test was as previously described (page 48). Red blood cells were sensitized with an extract

of P. multocida strain #37 (a type A strain from human sputum). Sensitized cells were prepared once a week and could be used satisfactorily for 5 or 6 days. After that time the cells tended to hemolyse. A known positive type A serum was included in each day's tests as a control. Two dilutions of each serum, 1/20 and 1/80, were tested in the screening test. All sera giving positive reactions were tested further to determine the titer of antibodies. The positive sera were also tested with a suspension of normal human O cells (uncoated) to rule out the possibility of non-specific agglutination of erythrocytes.

Results:

Antibodies which reacted with type A P.multocida were demonstrated in 18 of the 1000 sera tested. However, 2 of the positive sera were repeat specimens from patients previously showing positive titers. The patients had been discharged and re-admitted to hospital within the three month period in which the survey was carried out. The titer of antibodies in the 18 sera ranged from 1/40 - 1/640.

Sputum, or nose and throat swabs, were obtained for culture from 8 of the 16 patients. Pasteurella multocida was recovered from one sputum specimen - a single colony was found amongst mixed respiratory flora in anaerobic culture on blood agar.

Information about the patients including clinical diagnosis and history of contact with animals, together with results of hemagglutination tests and cultures is presented in table XV.

Discussion

The significance of a positive reaction in a serological procedure such as this will depend on the specificity of the test. The evidence presented in part IV suggests that the specificity of the hemagglutination

Table XV

Positive Serology in Patients Not Previously Known to Have P.multocida infection

Case #	Age	Sex	Date of test	Titer	Culture for P. multocida	Diagnosis	Contact with animals
1.	54	M	Feb.20/63	1/160	-	hypertension	farmer
2.	70	M	Feb.25/63	1/160	nose & throat - negative sputum - negative	ankylosing spondylitis	farmer
3.	78	M	Mar. 7/63	1/80	nose & throat - negative	bronchial carcinoma	farmer
4.	76	M	Mar. 8/63	1/320	sputum - negative	carcinoma of stomach	left farm 23 years ago
5.	40	M	Mar. 8/63	1/160	-	psychiatric	works on poultry farm
6.	62	M	Mar.19/63	1/160	-	cerebral vascular disease	left farm 17 years ago, now visits occasionally
7.	56	F	Mar.19/63	1/160	-	hepatic degeneration	housewife - no contact with animals
8.	61	M	Mar.20/63	1/160	sputum - negative	myocardial infarct	left farm 5 years ago
9.	70	M	Mar.20/63	1/640	sputum - scant P.multocida	osteo arthritis	retired farmer
10.	45	M	Mar.28/63	1/80	-	hemorrhoids	left farm 1 year ago
11.	62	M	Apr. 2/63	1/160	-	arteriosclerosis	rural resident*
12.	79	M	Apr. 8/63	1/160	-	dermatitis	rural resident*
13.	69	M	Apr.19/63	1/80	sputum - negative	bronchial asthma	left farm 3 years ago
			May 2/63	1/160	sputum - negative		
14.	33	M	Apr.22/63	1/40	-	pancreatitis	scaler in packing plant
15.	71	M	Apr.23/63	1/160	sputum - negative	chronic bronchitis	left farm 7 years ago
			May 3/63	1/80			
16.	71	M	May 9/63	1/160	sputum - negative	emphysema	farmer

*. Information about contact with animals could not be obtained

test in typing of P.multocida is of a high order, yet it does not necessarily follow that the application of the same technique to the identification of antibodies will be so specific. It is feasible that antigenic relationships might exist between P.multocida and certain other organisms, for instance, certain members of the Enterobacteriaceae, so that an antigenic stimulus provided by the latter could result in production of antibodies which would react with the former in a serological test. However the absence of cross reaction in the HA test between P.multocida and closely related species such as P.hemolytica, P.pseudotuberculosis, P.pfaffi suggests that cross reaction with more distantly related organisms is unlikely. Furthermore, if such a reaction did occur it would probably be at a very low level i.e. a titer of less than 1/40.

The positive reactions given by the sera of these 16 patients is therefore, I believe, strong indication of past or present infection with P.multocida. (The term infection is used in the sense defined by Dubos, 1958 (55) - the presence of micro-organisms in the tissues whether or not it results in detectable pathologic effect. There is no evidence to suggest that P.multocida contributed to the disease entities in any of these patients.) The organism was isolated from only 1 of 8 patients on whom cultural investigations were carried out. Failure to demonstrate the organisms however, does not mean it was necessarily absent in each of the 7 other patients. A single sampling of sputum might well fail to reveal an organism existing as a commensal in the respiratory tract.

The finding of 1.6% sera positive against type A P.multocida was unexpected. This relatively high incidence supports the contention that human infection with P.multocida is not a rare occurrence. If the sera had also been tested against a type D antigen it is quite likely that the incidence of this selected population showing antibodies to P.multocida would be over 2%.

The present or past association with farm animals or poultry is a common feature which seems significant in the history of these patients. Most of the patients were farmers or had farmed before moving to the city, but the period since leaving the farm was in some instances quite considerable, 23 years in one, 17 years in another. Because the background of Alberta is predominantly agricultural, the majority of patients in the University Hospital might also give a history of present or past association with farms. Therefore, a control group of 16 similar patients with negative HA tests was questioned regarding contact with farm animals. The control group was also drawn from patients over 30 years of age admitted to the Mewburn Pavilion or the medical wards of the University Hospital and was matched in regard to sex and age distribution with the test group. (table XVII). Information about the control group is given in table XVI. The proportion of persons having recent contact (i.e.within 10 years) with farm animals is considerably higher in the test group (12/14) than in the control group (5/16). (table XVII).

Table XVI - Control Group - History of Contact with Farm Animals

Control #	Age	Sex	Occupation	Contact with farm animals
1	73	M	Retired - car dealer	worked on farm for 6 months in 1919.
2	78	M	Retired - railway worker	worked on farm for 12 months in 1930's.
3	49	M	Truck driver	None
4	59	F	Housewife	born on farm - left at age 2.
5	75	M	Retired	None
6	73	M	Retired - farmer	Left farm 10 years ago
7	61	M	Farmer	Cows, pigs
8	47	M	Farmer	Cows, pigs, chicken
9	68	M	Janitor	Home in country, has 1 pig and 4 chickens
10	69	M	Retired - farmer	left farm 4 years ago
11	33	M	Carpenter	None
12	57	M	Maintenance	None
13	66	M	Truck driver	worked on farm 1920-22
14	74	M	Retired - farmer	left farm 14 years ago
15	64	M	Contractor	lived on farm 1910-1920
16	67	M	Commissionaire	worked on farm for 3 years before 1920.

Table XVII - Comparison of Test and Control Groups as to Age, Sex and History of Contact with Farm Animals

Total	Grouping / Series	Age					Sex		Contact with farm animals within 10 years
		31-40	41-50	51-60	61-70	Over 70	M	F	
16	Test	2	1	2	6	5	15	1	12/14
16	Control	1	2	2	6	5	15	1	5/16

Part VI. General Discussion

Detailed study of 59 human strains of Pasteurella multocida revealed the following features as constant and characteristic of the group - grey translucent colony on blood agar, coccobacilliary appearance in smears, distinctive odor, failure of growth on MacConkey's agar, fermentation of glucose, production of indol and in vitro sensitivity to penicillin. Two additional features exhibited by almost all strains were fermentation of sucrose and pathogenicity to mice. By means of these features strains of P. multocida are readily recognized and identified. Differentiation from other species of Pasteurella presents no problem. Strains of P. hemolytica P. pseudotuberculosis and the Pasteurella-like organisms included in this study for comparison showed a low degree of similarity to P. multocida.

The existence of subgroups among human strains of P. multocida was demonstrated both by serological methods and by the methods of numerical taxonomy. The strains of this series fell into 4 groups on the basis of numerical methods. These were labelled groups I, II, III and IV for the purposes of this investigation. Group I, the largest, consisted of strains predominantly of respiratory origin. The organisms were capsulated and tended to produce a mucoid growth on culture. Group II strains, also recovered mainly from respiratory tract infection, were capsulated but did not produce a mucoid growth. Strains arising from dog and cat bite infections were non-capsulated and fell into Groups III and IV.

By means of the hemagglutination test two serological types were demonstrated, Carter's type A and D, but many strains could not be classified due to lack of type specific capsular substance. Strains serologically type A fell into group I of the computer grouping, type D strains were included in group II. This close correlation between results of typing by two very different methods is evidence for the validity of

each method and suggests that the groupings obtained represent natural and not artificial affinities.

The status of Carter type B strains in relation to numerical methods of classification could not be determined. Only one type B strain was included in the present series and it fell into group II. Obviously more type B strains must be studied in order to determine if they too form a distinct group by numerical methods.

Two distinct groups were revealed among non-capsulated strains by the methods of numerical analysis but not by presently accepted serological tests. The differentiation was mainly due to differences in fermentative properties. All strains of group III fermented mannitol and the majority fermented sorbitol, xylose and glycerol. Strains of group IV failed to attack these carbohydrates. All strains derived from cat bites fall into group III, strains originating from dogs appear in both groups III and IV.

Differences between dog and cat strains of Pasteurella multocida have been noted by several workers. Talbot and Sneath (7), in their taxonomic study of strains isolated from human sources, found that strains derived from cat bites showed a high degree of similarity with each other while dog strains were more variable. The results of the present study are in agreement with their findings. Smith (Smith,1958 (22) and Smith, 1959 (56)) felt he could distinguish between dog and cat strains on the basis of cultural and biochemical differences. Dog strains usually fermented maltose and trehalose but not xylose, sorbitol nor mannitol; cat strains, on the other hand, fermented xylose, sorbitol and mannitol. Bain 1957 (57) suggests that dog and cat strains probably constitute a group quite distinct from strains derived from farm animals. However, he does not record differences within the "dog and cat" group.

It is apparent from this study that strains from dogs and cats fall into two distinct groups but it is unwarranted, at present, to consider one group as constituting a cat variety and the other, a dog variety. In this series the ten strains of canine origin were evenly split between the two groups indicating either that the "cat" variety is readily communicated from cats to dogs or that both groups exist naturally in the dog population.

The hemagglutination technique of Carter has provided useful information in classification of animal strains of Pasteurella multocida but shortcomings and inadequacies of the method are evident. A large number of strains of known pathologic significance cannot be typed. Approximately 40% of the strains of this series could not be classified by the hemagglutination test and yet, as just indicated, two distinct sub-groups exist within this unclassified group.

Some technical difficulties in carrying out the hemagglutination test have already been discussed. One problem appears to be due to variation in the amount of type specific antigen present in crude extracts of the organisms. This occurs particularly with type A strains. Another difficulty arises because of the marked tendency of some capsulated strains of P.multocida to dissociate in culture giving rise to non-capsulated variants. Care must be taken to ensure that capsulated organisms are selected for hemagglutination test. They can be picked out readily by examining serum agar cultures illuminated by oblique, transmitted light. Colonies of capsulated organisms usually appear iridescent, while those arising from non-capsulated variants are blue or grey and non-iridescent.

Carter and Bain (21) have found that strains identified as type A vary greatly in animal origin, colonial appearance, virulence for mice and serological reactivity. They suggest therefore that subtypes probably exist in this category. The dendrogram on page 34a shows that group I

(which includes 27 of the 29 type A strains of this series) is less homogeneous than the other 3 groups also indicating considerable variation within type A.

A suspected subtype of type B has been reported by Bain and Knox 1961 (25). An organism isolated from an ox in Australia was identified as type B by hemagglutination tests but it differed in biochemical and animal pathogenicity tests from classical type B strain found in Asia. Antigenic analysis showed that while the polysaccharide and protein antigens of the Australian and Asian strains were similar, the purified lipopolysaccharide was different chemically, biologically and serologically.

Thus it is evident that typing by means of the hemagglutination test does not go far enough. Sub-types exist which are not revealed by this method. The lack of antigenic homogeneity in presently recognized types was brought out in the fluorecent antibody studies of this investigation. Absorption of typing sera for use in FA tests showed that at least two sub-types exist in both type A and type D. Further work must be done to determine the number, distribution and significance of these sub-types.

It is quite likely that the sub-types revealed in FA studies correspond to the somatic antigen relationships demonstrated in recent work by Namioka and Murata, 1961 (58). These workers treated organisms with 1N HCl and found that the antigens exposed could be used to subdivide Carter's types A, B and D. Ten different O groups have now been distinguished by Namioka and Bruner, 1963 (59) and by correlation of Carter's capsule types and the O groups, 12 serotypes are delineated. Namioka and Bruner conclude that the present capsular types should be considered as groups, with the organisms being further divided into types on the basis of their O antigens.

The characteristics of strains isolated from respiratory tract, CSF and ear suggest that these organisms were derived from farm animals. Of 39 such strains in this series 36 were identified as type A or D by the hemagglutination test. These are the types that are prevalent in cattle, pigs and poultry (21). No history was available from 6 of the 36 patients from whom the type A and D strains were isolated but of the remaining 30, 20 had a definite history of contact with farm animals, 8 were known to live in rural areas although information about contact with farm animals was not obtainable, and 2 claimed to have had no contact with animals. Thus the great majority of these patients had ample opportunity for exposure to possible reservoirs of infection.

It is difficult to predict with present information which animal species is the likely source of a particular infection. The relative distribution of type A and D in cows, pigs and poultry in Canada is shown in the following figures compiled from data published by Carter, 1957(60), 1959 (61). (The strains were obtained mainly from Alberta and Ontario.)

	Type	
Source	A	D
Cow	39	1
Pig	44	38
Fowl	16	4

These figures suggest that the source of a type D strain would probably be a pig, but that type A strains could be derived from cow, pig or fowl. More precise epidemiological investigation of the source

of a P. multocida infection must await more precise serological characterization of the responsible strain. The identification of serotypes as described by Namioka and Bruner (59) may be helpful.

The significance of Pasteurella multocida in human respiratory infection is difficult to assess. Most isolations have been from chronic bronchial infections such as bronchiectasis or chronic bronchitis, diseases in which a great variety of bacteria may be found. However, repeated recovery of P. multocida as the predominant bacterial species in the respiratory flora would suggest that, in these cases at least, it is significant. The high titer of antibodies to P. multocida which may be found in some respiratory infections also indicates that the organism is contributing to the disease process. In this connection it should be emphasized that simple agglutination tests with the patient's serum and his own organism may fail to demonstrate the presence of antibodies. Strains recovered from respiratory infections are generally capsulated and thus inagglutinable in specific antisera. Antibodies may be demonstrated however by the hemagglutination test.

A serological survey of "normal" patients admitted to a large Edmonton hospital revealed that past or present infection with P.multocida is not uncommon among this group. Again a dominant feature of the history of the patients with positive titers was association with farm animals. These findings tend to indicate that P.multocida can exist in the normal respiratory tract and can probably be carried for some time.

Part VII. Conclusions

Classification of human strains of Pasteurella multocida by the methods of numerical taxonomy revealed four sub-groups. Groups I and II consisted of capsulated strains isolated mainly from the respiratory tract; groups III and IV were made up of non-capsulated strains predominantly of animal bite origin.

By means of the hemagglutination test of Carter, two serological types (A and D) were demonstrated, the rest of the strains could not be classified due to lack of specific capsular substance. Types A and D corresponded closely to groups I and II respectively of the numerical grouping. The presence of at least two sub-types within both types A and D was indicated by fluorescent antibody studies.

Pasteurella multocida strains isolated from human respiratory tract were of the same serological types as those found in cattle, pigs and fowl in Canada, suggesting that these animals are the source of the organism in most cases of human respiratory infection.

The presence of specific antibodies to P. multocida was demonstrated in patients with multocida infection of the respiratory tract and in 1.6% of a group of 1000 adult medical patients admitted to a local hospital.

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Appendices

Appendix 1.

Media Recipes

Serum Tryptose Agar

5% horse or bovine serum is added to Bacto Tryptose Agar Medium (Difco)

Formula of Bacto Tryptose Agar is:

Bacto-Tryptose	20 g.
Bacto-Dextrose	1 g.
Sodium Chloride	5 g.
Bacto-Agar	15 g.
Distilled Water to	1000 ml.

Cystine Trypticase Agar (BBL Lot #101622)

Cystine	0.5 g.
Trypticase	20.0 g.
Agar	2.5 g.
Sodium Chloride	5.0 g.
Sodium Sulfite	0.5 g.
Phenol Red	0.017 g.
Distilled Water to	1000 ml.

Enriched Peptone Water Medium

Trypticase (BBL)	10 g.
Proteose peptone #3 (Difco)	10 g.
Sodium chloride	5 g.
Distilled Water to	1000 ml.

Appendix II

Cultural and Serological Characteristics of Pasteurella-like Strains Isolated from Human Sputum.

Culture of sputum occasionally yields colonies of Gram negative bacilli which cannot be readily classified. Three such organisms, isolated in the Provincial Laboratory in Edmonton were initially picked out and investigated because they resembled P.multocida in colonial appearance. Further study, however, revealed that these organisms differed from P.multocida in several important respects. They were included in this investigation to determine their relationship with P.multocida and with each other.

The characteristics of these strains were as follows:-

Morphology: Considerable variation in size, shape and evenness of staining was noted. The organisms tended to be larger and more definitely rod shaped than P.multocida. They were Gram negative but often stained unevenly giving a vacuolated appearance. Some bipolar staining was noted. Two strains were non-capsulated, the third (strain 70) showed occasional capsulated organisms.

Cultural Characteristics: All 3 strains produced small grey translucent colonies on blood agar. No distinct hemolysis occurred but the medium under the colonies became discolored on continued incubation. The organisms tended to be more fastidious than P.multocida, only slight growth occurred on media without added serum or blood. No growth was produced on MacConkey. Cultures of the Pasteurella-like strains did not have the characteristic odor of P. multocida. Cultures soon became non-viable when left at room temperature for more than two or three days.

Biochemical Features: All strains produced acid in glucose, sucrose, galactose, maltose, and dextrin. Two strains fermented mannitol and levulose. Sorbitol, xylose, adonitol, inositol, arabinose, dulcite, salicin, lactose, raffinose, trehalose, mannose, rhamnose and glycerol were not fermented.

A striking feature was the rapid breakdown of urea; positive reactions occurring within 1 hour on urea agar slants. All strains produced oxidase and reduced nitrates. Indol, methyl red and Voges Proskauer reactions were negative, gelatin was not liquified, citrate was not utilized. Two strains produced H_2S. All strains were catalase negative.

Antibiotic Sensitivity: All strains were sensitive to penicillin, erythromycin, chloromycetin, tetracycline and streptomycin and resistant to novobiocin by the disc method.

Mouse pathogenicity: The strains proved to be non-pathogenic to mice.

Serological Tests: An antiserum to Pasteurella-like strain #70 was prepared by injecting a formalin killed saline suspension of organisms into a rabbit. The results of agglutination tests with this serum and various organisms are shown in Table XVIII.

TABLE XVIII.

Agglutination Tests with Antisera to "Pasteurella-like" strains

Strain #	Organism	Titer
70	Pasteurella-like (homologous)	1/320
68	Pasteurella-like	1/160
69	Pasteurella-like	1/320
67	*P.pseudotuberculosis*	-
66	*P.hemolytica*	1/160
62	*P.multocida* (cat)	-

The Pasteurella-like strains were not agglutinated by an antiserum prepared against a dog bite strain of *P.multocida*. Hemagglutination tests with *P.multocida* Type A, B and D antisera were negative.

Comments:

The three Pasteurella-like strains are closely related to each other and appear as a separate group in the dendrogram on page 34a. The strains show a low level of similarity to P.pseudotuberculosis and to P.multocida but a high degree of similarity to P.hemolytica. The relationship with P.hemolytica is also revealed by agglutination tests. The Pasteurella-like strains are differentiated from P. multocida on the basis of their microscopic morphology, their tendency to die out fairly rapidly in culture, failure to produce indol, lack of pathogenicity to mice and their strong urease reaction. The characteristics of these organisms suggest they resemble closely the strains recently described by Henriksen and Jyssum (12). These workers proposed the name P.hemolytica var.ureae for the strains considering the differences from P.hemolytica insufficient to justify the creation of a new species. Jones, 1962 (62), however, suggests that species status be given this group with the name of P. ureae.

No reference has been found to the isolation of a similar organism from a natural animal source. Thus, at present, this group represents a member of the genus Pasteurella (organisms regarded as primarily pathogens of animals) which is in search of an animal host.